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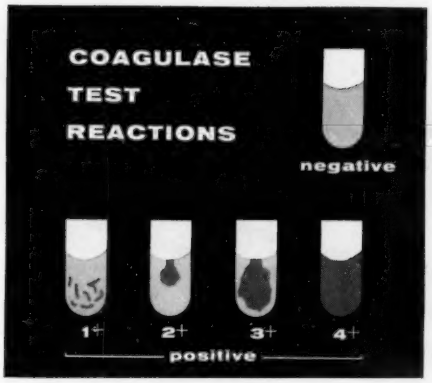

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1. Wootton, I. D. P.: Paper presented at International Congress of Clinical Chemistry, New York, Sept. 9, 1956.

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2. Brodie, J.; Sommerville, T., and Wilson, S. G. F.: Brit. M. J. *1*:667 (Mar. 24) 1956. 3. Conference on Staphylococcal Infections, New York Academy of Sciences, Feb. 16, 1956. 4. Boyd, H.: Am. J. M. Technol. *22*:232 (July-Aug.) 1956.

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5. Seegers, W. H.: J. Michigan M. Soc. *55*:272 (Mar.) 1956.

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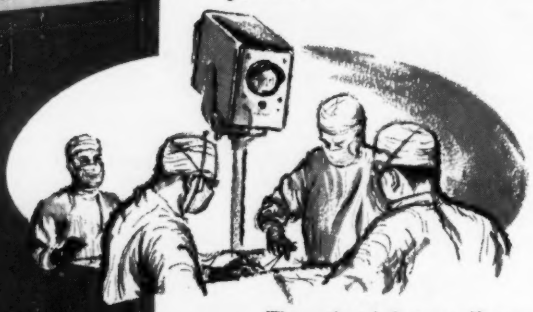

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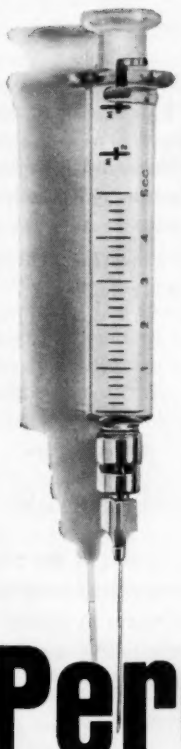

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*The American Foundation: Medical Research: A Midcentury Survey, Boston, Little, Brown and Company, 1955, vol. 1, p. XXXI.

**_Ibid._, p. 600.

THE RENAL RESPONSE IN MAN TO ACUTE EXPERIMENTAL RESPIRATORY ALKALOSIS AND ACIDOSIS[1]

By E. S. BARKER,[2,3] R. B. SINGER,[4] J. R. ELKINTON,[2] AND J. K. CLARK

(From the Renal Section and Chemical Section of the Department of Medicine, The Department of Research Medicine, and the Department of Biochemistry, the University of Pennsylvania School of Medicine, Philadelphia, Pa.)

(Submitted for publication August 7, 1956; accepted December 6, 1956)

The experimental results to be presented here deal with the renal component of the multiple effects in man of acute experimental respiratory alkalosis (hyperventilation) and acidosis (CO_2 inhalation). One aim of the experiments has been to define an integrated picture of the total body response to acute respiratory acid-base disturbances. A previous paper (1) contained a description of the effects observed in the same experiments on the composition of plasma and red cells, and a quantitative estimate of the exchanges of ions and water between red cells, plasma, extracellular fluids and a phase or phases outside the chloride space ("intracellular"). In the present report consideration is given to the changes in renal excretion and hemodynamics and an attempt is made to define more clearly certain mechanisms involved. Reference should be made to the previous report (1) for data on the blood or plasma changes; only a few of these data are included here when they are directly important to interpretation and renal effects. The present findings were previously reported in abstract (2, 3).

EXPERIMENTAL PROCEDURE AND CHEMICAL METHODS

Six normal male subjects actively hyperventilated and six inhaled CO_2 as described in detail in the preceding paper (1). Following control periods of 47 to 74 minutes' duration, hyperventilation was carried out for approximately 30 minutes in 5 of the 6 experiments, and for twice that period in the last experiment; 7.5 to 7.7 per cent CO_2 in air or oxygen was inhaled for 21 to 30 minutes. Measurements were continued in both types of experiments during subsequent recovery periods which ended 97 to 145 minutes after onset of the stimulus (designated time zero). Standard water loading was carried out before the experiments and continued throughout with water given in amounts equivalent to urine excreted. In respiratory acidosis a neutral or slightly alkaline control urine was considered desirable to facilitate observation of renal effects. Accordingly, in experiments 1 to 5 inclusive, the subjects were given 4.2 gm. $NaHCO_3$ (50 mEq.) by mouth at -95 to -150 minutes. As a control, in the sixth experiment NaCl was substituted for the $NaHCO_3$ and this experiment is not included in statistical analyses. Four additional control experiments were done in which the $NaHCO_3$ load was given, and observations were made for the usual experimental time (but with no respiratory stimulus) to indicate the effect of the loading procedure alone.

Renal clearances were determined by standard techniques and chemical methods (4, 5), with bladder catheterization and appropriate anaerobic collection of urine to prevent loss of CO_2. Changes in glomerular filtration rate were estimated from changes in endogenous creatinine clearance and effective renal plasma flow from p-aminohippurate (PAH) clearance.[5] Following an equilibration period of at least 45 minutes urine was collected for 3 periods before, 1 to 4 periods during, and 3 to 4 periods after the application of the stimulus (hyperventilation or CO_2 inhalation).

Total CO_2 was determined in the anaerobically collected urine by the manometric method of Van Slyke and Sendroy (6), urine pH at 37° C by means of the photocolorimetric method of Van Slyke, Weisiger, and Van Slyke (7), sodium and potassium with a Barclay internal standard flame photometer (8), chloride by the

[1] Laboratory facilities were aided by grants from the National Heart Institute of the United States Public Health Service (Grants H-405 and H-340), the Life Insurance Medical Research Fund, and the C. Mahlon Kline Fund for Development in the Department of Medicine.

[2] Established Investigator of the American Heart Association.

[3] In part during tenure of Post-doctorate Fellowship of the National Institutes of Health, United States Public Health Service.

[4] Present address: 501 Boylston Street, Boston, Mass.

[5] Excretion of anionic PAH^- in itself has some effect on urinary acid-base pattern. Administration was, however, at the same slow rate during both control and experimental periods and there were no significant *changes* in PAH excretion during either type of respiratory stimulus, when urinary acid-base changes were maximal. PAH could also form a buffer pair, but because it would be a strong acid buffer ($pK' = 3.83$) (13), such an effect must be very small.

Volhard-Harvey method (9), ammonia by the method of Folin and Bell (10) using a Klett-Summerson photometer, phosphate by the method of Lowry and Lopez (11), and titratable acidity by titrating to pH 7.4. The bicarbonate concentration was calculated from the total CO_2 content and pH of each urine specimen by use of factors for CO_2 solubility and pK' in urine as functions of urinary total cation concentration, derived from Sendroy, Seelig, and Van Slyke (12).

Methods of blood sampling and analyses were described in the previous paper (1).

CALCULATIONS

Urinary electrolytes in microequivalents per minute (μEq. per min.) are presented as excretion rates (UV) rather than clearances, thus permitting immediate comparison of cation-anion equivalents. The cations determined were sodium, potassium, and ammonium; the anions were chloride, bicarbonate, and (in part) phosphate.

Magnitude of acid-base disturbances ($\Delta HCO_3^-{}_{er}$). Under conditions termed a "steady state" the rate at which carbon dioxide from cellular metabolism is added to the extracellular fluid equals the rate at which it is lost from the body, and there is no resulting acid-base effect on the extracellular fluid. When the pulmonary component is disturbed, as by hyperventilation or CO_2 inhalation, a net quantity of CO_2 is removed from or added to the extracellular fluid. An acid-base effect is exerted by shifting the following relationship toward either the left or right:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \qquad (1)$$

This disturbance may be expressed in terms of equivalents as a change in HCO_3^-, and this change is proposed for the purposes of this paper as an indication of the magnitude of the acid-base disturbance. $\Delta HCO_3^-{}_{er}$ is defined as the change in total amount of bicarbonate in the extracellular fluid (plasma plus interstitial fluid) and in the red cells. It is desirable to include the red cells since they are intimately related by the chloride shift to the acid-base changes of respiratory disturbances. The method of determination, together with observed $\Delta HCO_3^-{}_{er}$ in the individual experiments, was given for a different purpose in the portion of the work previously published (1).

From Equation 1 it is evident that a change in H^+ equivalent to that in HCO_3^- must initially occur. However, at pH ranges compatible with life, free hydrogen ion *concentration* is always insignificant compared to the other ions. Accordingly, "buffering" mechanisms must accept the hydrogen ion in one type of disturbances or furnish it in the other.[6]

[6] The role of such mechanisms was estimated quantitatively, and it was noted that participation of some phase of body fluid outside the blood and typical interstitial fluid appears in these particular experiments to account for about two-thirds of the buffering of the hydrogen ion disturbance that would otherwise be present in the extracellular fluid (1).

Changes in rate of urinary hydrogen ion excretion (ΔUV_{H^+}). For the purpose of this paper a quantitative index of rate of H^+ excretion is defined as the sum of three components: NH_4^+ output plus titratable acid minus the HCO_3^- output. In the case of an alkaline urine the rate is a negative quantity dependent chiefly on the bicarbonate output while for urine of neutral or slightly acid pH values, all three terms are significant fractions of the total. Excretion of bicarbonate is equivalent in acid-base effect to the addition of hydrogen ion to the body and this method of calculating UV_{H^+} eliminates the necessity of treating HCO_3^- output as a separate factor. It is frequently not possible to distinguish, in the case of two separate body fluids, transfer of H^+ in one direction from transfer of HCO_3^- in the opposite direction.

In order to assess the rate of renal acid-base compensation to the respiratory disturbance it is desirable to consider the difference between UV_{H^+} observed and the UV_{H^+} which would have been present at the control rate. The *change* in urinary hydrogen ion excretion is therefore determined, thus,

$$\Delta UV_{H^+} = \Delta UV_{NH_4^+} + \Delta UV_{TA} - \Delta UV_{HCO_3^-} \qquad (2)$$

This consideration of ΔUV_{H^+} rather than UV_{H^+} in absolute terms also avoids arbitrary decisions on what constitutes a "neutral" urine as related to an extracellular fluid pH about 7.4 and a usual dietary intake and metabolism which, uncompensated, would produce a metabolic acidosis.

It should be noted that under these experimental conditions, once hyperventilation or CO_2 inhalation ceases the acid-base disturbance is rapidly corrected by respiratory adjustments. Therefore the most meaningful indications of urinary adjustments appear to be the peak ΔUV_{H^+} achieved and the cumulative change to the time the respiratory stimulus was discontinued.

RESULTS

The results are presented in Tables I A, I B, II and III and in Figures 1 through 4 and were evaluated by standard statistical methods. The average control data are included in the figures and the average changes, variability (standard error) and probability of chance occurrence are shown below the graphic part of each figure. Changes in urinary excretion rates during respiratory alkalosis or acidosis as mentioned hereafter are the mean change (from control rates) observed in the last urine collection period during hyperventilation or CO_2 inhalation.

Respiratory alkalosis

The magnitude of the acid-base disturbance was indicated by an average $\Delta HCO_3^-{}_{er}$ of -135 mEq. observed at the end of the period of hyperventila-

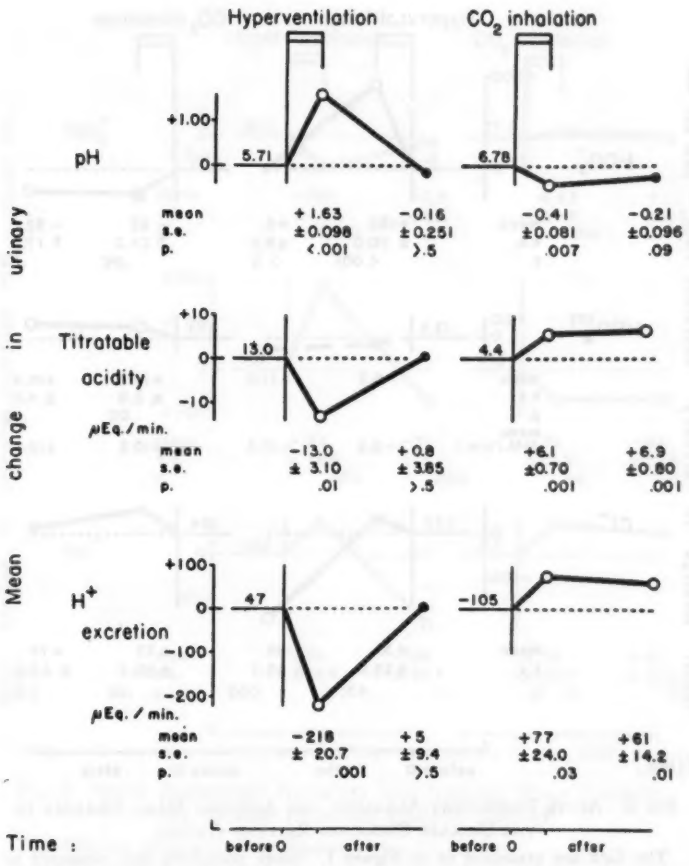

FIG. 1. ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS: MEAN CHANGES IN URINARY pH AND THE EXCRETION OF TITRATABLE ACID AND HYDROGEN ION

The mean for each group of changes from the individual mean control values is plotted for the end of the period of stimulus and the end of the experiment. The values for the mean change, its standard error, and the probability of chance occurrence are given below the curves. The mean changes that are statistically significant ($p = 0.05$ or less) are represented by open circles. The abscissae represent time before, during, and after the stimulus. The mean of the control values is given on the horizontal axis. Hydrogen ion excretion is defined as the sum of outputs of ammonium plus titratable acidity minus bicarbonate (see Equation 2 in text).

tion. The rate of renal adjustment to the disturbance by retaining H^+ ion as compared to control H^+ output (ΔUV_{H^+} by Equation 2) averaged 218 μEq. per minute for the final period during hyperventilation. During the 30 minutes of the stimulus an average of 5.9 mEq. H^+ had been retained in this way. During hyperventilation the pH of the urine rose (mean = + 1.63 pH units) and titratable acidity fell (− 13.0 μEq. per min.), both returning to the control values during the recovery period after hyperventilation. Bicarbonate excretion was markedly increased (+ 183 μEq. per min.) and ammonium ion excretion decreased (− 30 μEq. per min.); each subsequently re-

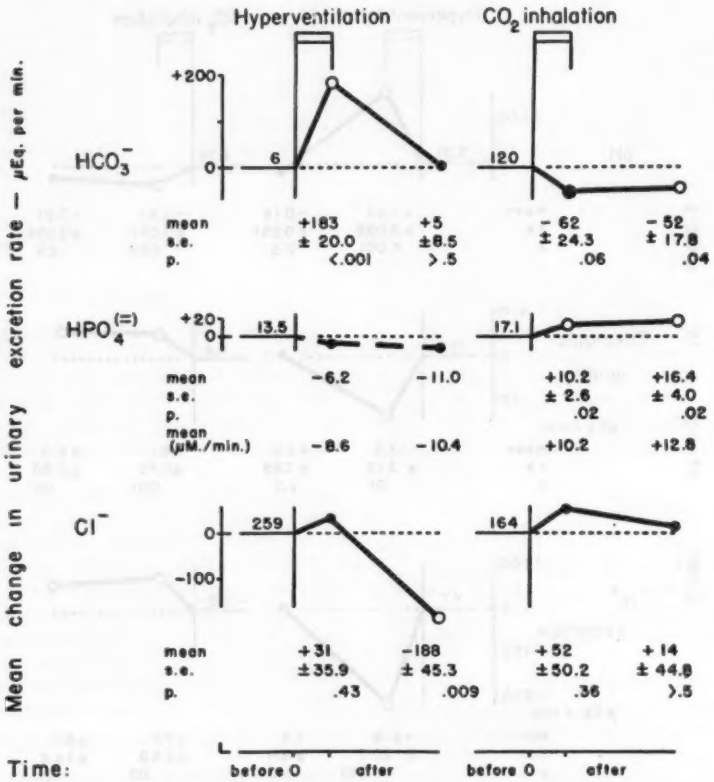

FIG. 2. ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS: MEAN CHANGES IN THE URINARY EXCRETION RATES OF ANIONS

The data are presented as in Figure 1. Since phosphate was measured in only two of the hyperventilation experiments, it is shown with a dotted line and statistical analysis omitted. The symbol $HPO_4^{(=)}$ indicates phosphate both as $HPO_4^=$ and $H_2PO_4^-$.

turned to control rates. Potassium excretion increased markedly (+ 183 μEq. per min.), while neither sodium nor chloride excretion showed a statistically significant change. In the recovery period excretion rates fell significantly below control for each of these ions (mean changes in rate per min.: K^+ − 71 μEq., Na^+ − 155 μEq., Cl^- − 188 μEq.).

Respiratory acidosis

It is clearly shown by comparison of the magnitude of the estimated acid-base disturbance ($\Delta HCO_{3er}^- = 26$ mEq.), or of plasma acid-base data (1), that CO_2 inhalation was a milder acid-base disturbance than hyperventilation. It is difficult to achieve a more severe acute experimental respiratory acidosis without undesirable side effects. Changes in urinary findings were correspondingly smaller during CO_2 inhalation than those observed during hyperventilation. ΔUV_{H^+} averaged 77 μEq. per minute and cumulative H^+ eliminated during the stimulus was 1.9 mEq. Urinary pH fell (− 0.41 pH units) and titratable acidity increased (+ 6.1 μEq. per min.). Bicarbonate excretion decreased (− 62 μEq. per min.) in spite of a considerable increase in filtered load that resulted from the increased plasma concentration during CO_2 inhalation. The change in bicarbonate reabsorption (+ 310 μEq. per min.) is accordingly significant ($p = 0.03$). Thus a

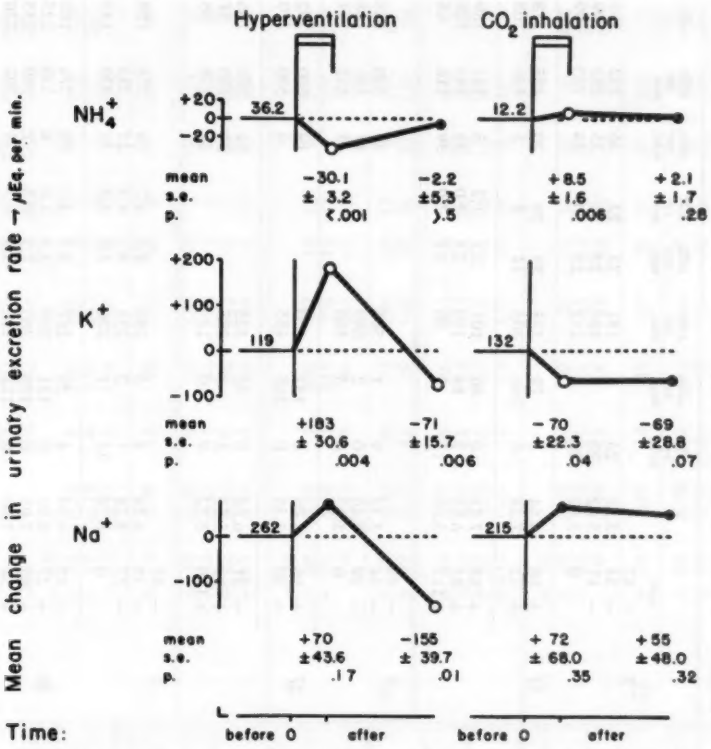

FIG. 3. ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS: MEAN CHANGES IN THE URINARY EXCRETION RATES OF CATIONS

The data are presented as in Figure 1.

real physiologic response in bicarbonate regulation is evident, although if changes in excretion alone are considered the results just fail ($p = 0.06$) to show "statistical significance." Excretion of ammonium ion increased (+ 8.5 μEq. per min.) and that of potassium decreased (− 70 μEq. per min.). Neither sodium nor chloride output showed statistically significant change. Phosphate excretion was increased (+ 10 μEq. per min.).

The small oral dose of sodium bicarbonate given preceding the respiratory acidosis experiments established a "baseline" (a neutral or alkaline urine) upon which the effect of the CO_2 inhalation could be more readily determined. Effects of the bicarbonate dose are evident in differences between the average control values in the respiratory alkalosis and acidosis experiments as shown in Figures 1 to 4. Four additional experiments

	PLASMA pH	URINARY HCO_3^- EXCRETION	TUBULAR HCO_3^- REABSORPTION	PLASMA pCO_2
RESPIRATORY ACIDOSIS	↓	↓	↑	↑
RESPIRATORY ALKALOSIS	↑	↑	↓	↓
METABOLIC ($NaHCO_3$ ADMIN) ALKALOSIS	↑	↑	↑	↑

FIG. 4. CHANGES IN $NaHCO_3$ EXCRETION, REABSORPTION AND RELATED EXTRACELLULAR FLUID (PLASMA) FACTORS IN ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS AND IN METABOLIC ALKALOSIS

Direction of change in response to the stimulus is indicated by the arrows. For each type of disturbance the plasma HCO_3^- concentration changed in the same direction as the PCO_2. The rate of filtration of HCO_3^- (or HCO_3^- load) also changed in this direction.

TABLE I A

Respiratory alkalosis: Effects of hyperventilation on urinary pH, titratable acidity, and excretion of electrolytes

Expt. and duration stimulus in min.	Time*	pH	Titr. acid.	HCO_3^-	Cl^-	NH_4^+	Na^+	K^+
	min.		*μEq. per min.*	*μEq. per min.*	*μEq. per min.*	*μEq. per min.*	*μEq. per min.*	*μEq. per min.*
1	− 48							
	0	6.3		17	245	26	217	139
29	+ 29	7.8		251	242	7	218	414
	+ 61	7.6		104	79	8	65	189
	+ 89	7.2		123	64	13	90	151
	+118	7.2		64	56	10	89	97
3	− 53							
	− 34	5.70	10	8	195	42	230	146
	− 20	5.80	6	11	165	38	210	127
	0	5.88	5	11	172	35	225	123
30	+ 14	7.00	0	111	325	19	520	232
	+ 30	7.38	0	184	240	4	420	272
	+ 45	7.01	0	45	81	2	147	143
	+ 60	5.7	12	2	76	34	163	126
	+ 90	5.5	10	2	37	34	139	37
	+121	5.2	17	0	34	50	143	22
4	− 74							
	− 53	5.30	22	1	135	48	127	84
	− 37	5.39	18	2	116	54	104	92
	0	5.40	18	1	80	48	93	60
37	+ 22	6.77	4	64	170	24	160	167
	+ 37	7.20	0	171	251	13	214	332
	+ 55	—	4	16	72	5	55	130
	+ 86	5.34	10	0	39	27	31	60
	+117	5.2	15	0	34	31	32	47

Expt. and duration stimulus in min.	Time*	pH	Titr. acid.	HCO_3^-	Cl^-	Phosphate		NH_4^+	Na^+	K^+
	min.		*μEq. per min.*	*μEq. per min.*	*μEq. per min.*	*μEq. per min.*	*μM per min.*	*μEq. per min.*	*μEq. per min.*	*μEq. per min.*
5	− 47									
	− 33	5.57	23	4	187	23	22	36	252	105
	− 17	5.62	20	8	193	22	21	35	252	99
	0	5.62	22	7	212	21	20	35	263	99
32	+ 16	6.64	5	79	335	28	20	20	455	178
	+ 32	7.28	0	166	305	14	8	5	440	208
	+ 53	7.21	0	80	101	2.1	1.2	5	143	189
	+ 83	6.16	7	14	110	2.4	2.0	22	143	108
	+117	5.60	11	5	70	3.1	2.9	39	116	65
6	− 49									
	− 34	5.32	9	1	565			39	520	148
	− 19	5.40	10	2	640			47	580	168
	0	5.62	6	3	510			45	455	144
27	+ 16	7.04	0	132	610			19	600	292
	+ 30	7.35	0	248	485			6	500	370
	+ 51	6.68	0	36	325			14	258	186
	+ 82	5.55	5	2	285			34	288	57
	+116	5.22	9	0	176			41	178	38
7	− 55									
	− 38	6.24	4	3	258	4.6	4.7	18	278	109
	− 23	5.78	7	2	255	4.7	4.3	22	253	—
	0	5.28	15	1	250	5.0	4.8	31	220	118
58	+ 15	6.38	4	29	267	5.5	4.3	22	274	163
	+ 27	6.91	0	121	289	2.5	1.6	4	330	228
	+ 40	7.01	0	128	280	1.3	0.8	2	280	233
	+ 58	7.06	0	117	218	0.5	0.3	2	202	220
	+ 77	6.84	0	36	138	0.4	0.3	4	98	135
	+108	5.12	11	0	86	0.5	0.3	24	92	52
	+144	4.86	17	0	56	1.9	1.9	33	83	24

* Data are expressed per individual periods which end at the time indicated, measured from the start of hyperventilation. Values obtained during hyperventilation are separated by horizontal single lines.

TABLE I B

Respiratory acidosis: Effects of CO_2 inhalation on urinary pH, titratable acidity and excretion of electrolytes

Expt. and duration stimulus in min.	Time*	pH	Titr. acid.	HCO_3^-	Cl^-	Phosphate		NH_4^+	Na^+	K^+
	min.		μEq. per min.	μEq. per min.	μEq. per min.	μEq. per min.	μM per min.	μEq. per min.	μEq. per min.	μEq. per min.
1	− 37									
	− 25	7.00	7	56	296	29	18	23	352	83
	− 13	7.06	—	58	222	27	16	—	275	69
	+ 4	6.91	6	70	227	34	22	26	300	83
30	+ 14	6.50	6	50	243	36	27	39	373	49
	+ 33	6.34	14	60	469	48	39	36	634	39
	+ 48	6.52	9	74	400	47	35	22	592	42
	+ 68	6.52	12	25	292	38	28	26	400	27
	+ 97	6.18	21	9	217	36	30	42	311	16
2	− 57		†							
	− 41	6.93	4	245	164	23	15	10	275	199
	− 17	6.86	4	221	142	23	15	10	258	177
	− 2	6.83	—	181	133	—	—	9	235	156
27	+ 14	6.74	6	152	138	24	16	13	237	113
	+ 30	6.49	9	84	123	27	20	14	190	74
	+ 45	6.69	8	106	151	34	24	16	240	84
	+ 67	6.57	9	95	112	30	22	17	222	73
	+ 90	6.60	11	125	120	37	27	16	264	125
	+121	6.72	10	130	124	40	28	13	239	107
3	− 47		†							
	− 32	6.61	4	59	100	14	10	14	167	48
	− 16	6.82	2	84	104	9	6	11	182	64
	− 1	6.76	2	109	135	10	6	10	233	82
29	+ 13	6.97	2	104	152	14	9	9	252	85
	+ 29	6.59	7	70	214	22	16	17	282	58
	+ 44	6.64	7	67	209	24	17	14	280	64
	+ 63	6.73	8	111	317	31	21	12	425	90
	+ 97	6.73	8	124	352	33	23	12	480	100
	+129	6.73	9	97	305	37	25	13	415	92
4	− 47									
	− 32	6.72	4	116	137	4	3	9	186	129
	− 17	6.78	4	137	143	5	4	7	197	167
	− 2	6.73	6	119	169	6	4	7	216	155
21	+ 22	6.29	12	44	170	10	8	17	191	83
	+ 37	6.40	9	53	161	10	8	16	238	55
	+ 53	6.45	11	56	153	9	7	9	245	47
	+ 82	6.46	6	56	112	10	7	13	211	32
	+111	6.29	10	41	101	18	15	15	173	23
5	− 50									
	− 36	6.68	3	139	182	18	12	8	211	221
	− 20	6.58	2	111	158	15	11	7	181	189
	0	6.52	7	96	143	17	12	9	173	164
30	+ 15	6.46	6	73	124	22	17	9	157	116
	+ 30	6.17	11	29	100	29	24	20	138	58
	+ 44	6.27	20	36	150	43	35	20	193	68
	+ 61	6.45	18	53	144	46	36	13	206	86
	+ 92	6.51	16	67	160	51	38	12	250	82
	+122	6.57	14	62	135	39	28	8	223	62
6‡	− 51									
	− 35	5.88	12	10	56	11	10	40	33	105
	− 19	5.88	11	11	48	12	11	32	50	107
	0	5.94	13	14	58	14	13	32	56	118
22	+ 24	5.97	14	21	139	23	21	37	165	95
	+ 42	5.89	22	14	144	27	24	36	177	99
	+ 60	6.01	11	17	155	30	26	32	192	105
	+ 96	5.88	22	13	151	30	27	40	151	117
	+129	5.79	23	8	86	28	26	44	78	111

* Data are expressed per individual periods which end at the time indicated, measured from the start of CO_2 inhalation.
Values obtained during CO_2 inhalation are separated by horizontal single lines.
† Titratable acidity calculated from phosphate in Experiments 2 and 3.
‡ Experiment 6 is not included in statistical analyses. This experiment was done as a control, the urine being initially acid as explained in the text.

TABLE II

Respiratory alkalosis and acidosis: Calculated extracellular acid-base disturbance ($\Delta HCO_3^-{}_{ec}$) and renal compensation (ΔUV_{H^+})† at end of respiratory stimulus*

A. Respiratory alkalosis (hyperventilation)				B. Respiratory acidosis (CO_2 inhalation)			
		ΔUV_{H^+}				ΔUV_{H^+}	
Expt.	$\Delta HCO_3^-{}_{ec}$	Rate	Cumulative	Expt.	$\Delta HCO_3^-{}_{ec}$	Rate	Cumulative
	mEq.	*μEq. per min.*	*mEq.*		*mEq.*	*μEq. per min.*	*mEq.*
3	−141	−215	−5.0	1	+46	+ 20	+0.64
4	−189	−226	−5.6	2	+12	+142	+4.3
5	−125	−212	−5.0	3	+38	+ 23	+0.16
6	−152	−292	−6.7	4	+ 2	+ 97	+2.3
7	− 67	−146	−7.0	5	+31	+105	+2.2
Mean	−135	−218	−5.9	Mean	+26	+ 77	+1.9

* $\Delta HCO_3^-{}_{ec}$ indicates the total change in bicarbonate of the extracellular fluid (plasma plus interstitial fluid) and of the red cells (1).

† ΔUV_{H^+} indicates the change from control in urinary hydrogen ion excretion defined as the sum of ammonium plus titratable acid minus bicarbonate output (see Equation 2 in text).

were done without any disturbance of respiration to evaluate the effect of the oral bicarbonate alone. These experiments also provided a control for the acidosis series with respect to the effects of the diurnal "tide" and the water loading procedure. While slight apparent changes were present at the times that would correspond to the respiratory stimulus, none of them was statistically significant. Changes during the stimulus in the respiratory acidosis series which are significantly different from the preceding control values also differ significantly from the slight alterations at corresponding times in the control series. This is true for all of the variables except urinary pH and K^+ excretion, the differences of which were not quite significant at the 5 per cent level. During the comparatively longer recovery portion of the experiments, a tendency in the acidosis series to disappearance of the effects of the oral bicarbonate on the "baseline" would be indistinguishable from persistence of acidification of the urine as a compensatory mechanism to the respiratory disturbance. Accordingly, discussion of changes late in recovery is limited to respiratory alkalosis. Results are not reported in detail for the control series.

Renal hemodynamics and urinary volume

Measurements made of effective renal plasma flow, glomerular filtration rate, and rates of urine flow are not reported in detail. Significant changes were observed only with hyperventilation. Effective renal plasma flow was depressed from control at the end of these experiments, a change correlating with the excretion rate of sodium and chloride which were also significantly depressed at this time. Glomerular filtration rate was also decreased significantly at the end of the experiments, but also decreased somewhat during the hyperventilation, at a time when excretion of sodium and chloride and effective plasma flow were not significantly altered and if anything were a little increased. Changes in urine flow were probably more closely related to the water loading than to the experimental procedure.

DISCUSSION

Observation of renal acid-base adjustment

The kidney responds promptly to primary respiratory alterations of acid-base equilibrium. The renal adjustment is not a simple correction of the abnormality present, but may be considered as eventually producing an opposing "metabolic" (as contrasted to "respiratory") disturbance which may be measured as a change in the rate of hydrogen ion excretion (ΔUV_{H^+}). An increase in UV_{H^+} is equivalent to an increase in buffer base in the body. Singer and Hastings (14) suggested certain practical advantages in focussing attention on the buffer base concentration as a quantitative index of the metabolic or non-respiratory factor in acid-base equilibrium and pointed out that a pure respiratory disturbance (before various com-

TABLE III

Respiratory alkalosis and acidosis: Renal filtration, reabsorption and excretion of bicarbonate

Expt.	Time*	Urine flow (ml. per min.)	GFR (ml. per min.)	Plasma pH	Plasma PCO_2 (mm. Hg)	Plasma HCO_3^-† (mEq. per L.)	HCO_3^- Filt. (mEq. per min.)	HCO_3^- Excr. (mEq. per min.)	HCO_3^- Reabsorbed (mEq. per min.)	HCO_3^- Reabsorbed (mEq. per L. filtrate)
				A. Respiratory alkalosis—hyperventilation						
3	C*	10.4	125	7.38	43	24.9	3.11	.010	3.10	24.82
	S	5.0	109	7.62	19	19.2	2.09	.184	1.91	17.52
	R	1.9	124	7.40	42	25.4	3.15	0	3.15	25.42
4	C	6.6	104	7.35	43	23.6	2.45	.001	2.45	23.58
	S	6.2	108	7.65	15	16.5	1.78	.171	1.61	14.92
	R	0.5	80	7.43	37	24.4	1.95	0	1.95	24.41
5	C	12.1	122	7.39	43	25.6	3.13	.006	3.12	25.58
	S	5.3	87	7.63	19	20.2	1.76	.166	1.59	18.29
	R	9.3	118	7.44	39	25.7	3.03	.005	3.03	25.67
6	C	7.5	109	7.39	43	25.3	2.76	.002	2.76	25.28
	S	7.8	86	7.66	16	17.9	1.54	.248	1.29	15.00
	R	3.8	96	7.43	37	24.2	2.32	0	2.32	24.20
7	C	1.6	132	7.30	44	24.9	3.29	.002	3.29	24.89
	S	5.9	108	7.56	25	22.2	2.40	.117	2.28	21.13
	R	3.9	109	7.39	42	24.9	2.71	0	2.71	24.89
				B. Respiratory acidosis—CO_2 inhalation						
1	C	3.0	139	7.40	46	27.5	3.82	.061	3.76	27.05
	S	17.2	151	7.35	54	29.9	4.52	.060	4.46	29.53
	R	4.0	135	7.45	40	27.0	3.64	.010	3.64	26.97
2	C	18.2	126	7.46	40	27.8	3.50	.216	3.29	26.11
	S	16.4	127	7.40	47	28.7	3.64	.123	3.52	27.71
	R	17.2	131	7.43	40	26.5	3.47	.125	3.35	25.57
3	C	11.1	111	7.50	34	25.5	2.83	.084	2.75	24.76
	S	10.6	114	7.37	48	27.1	3.09	.070	3.02	26.49
	R	13.6	117	7.44	37	24.4	2.86	.111	2.74	23.42
4	C	16.7	115	7.44	41	26.7	3.07	.124	2.95	25.63
	S	14.8	117	7.34	51	27.0	3.16	.044	3.12	26.68
	R	15.8	116	7.48	34	24.6	2.85	.056	2.80	24.13
5	C	16.8	108	7.44	38	25.3	2.73	.115	2.62	24.26
	S	13.1	107	7.33	52	26.5	2.84	.029	2.81	26.18
	R	14.1	106	7.44	26	24.2	2.57	.053	2.51	23.68
6‡	C	12.1	102	7.32	49	24.7	2.52	.012	2.51	24.60
	S	15.3	110	7.26	62	27.1	2.98	.021	2.96	26.90
	R	14.7	106	7.37	45	25.5	2.70	.013	2.69	25.38

* Time of individual periods and respiratory stimuli is given in Table I A and I B.
C denotes average of the control periods.
S denotes the last period during the respiratory stimulus.
R denotes the last period for which complete data were available during recovery from the effects of the stimulus.

† The plasma concentration of HCO_3^- given and used to calculate HCO_3^- filtered is that observed (often at the end of the period) rather than an interpolated value for mid-period. No correction is made for serum water or Donnan factor.

‡ Experiment 6 during CO_2 inhalation was done as a control, the urine being initially acid as explained in the text.

pensations) causes no change in whole blood buffer base. Fuller and MacLeod (15) have calculated the rate of "total H^+ secretion" by adding HCO_3^- *reabsorption* to titratable acidity plus ammonium excretion rather than by subtracting HCO_3^- *excretion* as we do in calculating UV_{H^+}. The resulting index is quite different since our index indicates the net *effective acid-base adjust-*

ment accomplished by the over-all process of urine formation while theirs relates to the specific process of H^+ secretion by the tubular cells.[7]

In *respiratory alkalosis* the characteristic response is a decrease in H^+ output, measured principally by an increase of HCO_3^- with fixed cation in an alkaline urine (17–22). Associated with this is a decrease in output of ammonia and titratable acid. The resultant effect on total body fluids is a loss of buffer base, a further reduction of the already lowered HCO_3^- concentration and a return of pH toward the normal range. The accompanying predominant change in excretion of "fixed" ion in the first half hour of acute experiments is increased K^+ loss, rather than change in Na^+ or Cl^-. The duration of the experiments was such that cumulative excretion while the stimulus persisted had very little effect in changing extracellular or total body fluid buffer base. The renal compensation was therefore small compared to the rapid sharing of the effects of the stimulus by the various "buffer" mechanisms of the body previously estimated in detail (1). Calculations based on the data of other workers (23, 24) indicate that in more prolonged respiratory disturbances the major part of ΔHCO_{3er}^- may be compensated by cumulative urinary changes.

Renal compensation for acute respiratory alkalosis appears to be less efficient than that for acute metabolic alkalosis of the "electrolyte addition" type. In seven experiments in which we gave rapid intravenous infusions of hypertonic sodium bicarbonate (25, 26) the total dose of bicarbonate given was of the same order of magnitude as the ΔHCO_{3er}^- observed in respiratory alkalosis reported here. Yet, within the same time, cumulative ΔUV_{H^+} averaged 19.6 mEq., indicating more than three times the comparable renal compensation during respiratory alkalosis.[8] The difference may be related to the fact that this type of metabolic alkalosis (a simple excess of sodium and bicarbonate) may be corrected by the renal excretion of the ions present in excess.

The characteristic changes in *respiratory acidosis* are the opposite of those described for respiratory alkalosis (15, 17, 27). There is an increase in excretion rates of H^+, titratable acid and ammonia and a decrease in excretion of bicarbonate with fixed cation, especially potassium. The urine becomes more acid. Since the acid-base disturbance of acute experimental CO_2 inhalation is considerably milder than that of hyperventilation, the urinary changes are much smaller in absolute terms. Relative to the estimated acid-base disturbance (ΔHCO_{3er}^-), however, the renal response appears to be of the same order of magnitude. When the experiments were planned we postulated that it would be difficult to detect an increase in UV_{H^+} if the control urine were already acid. If the "basal" state of the kidney involves compensation for the tendency of the usual diet to produce a slight metabolic acidosis, it would scarcely be surprising that an acute respiratory acidosis of mild degree should fail to bring about a further detectable increase in UV_{H^+}. We believe that this is the explanation for the lack of response in urinary acid-base factors noted by Longson and Mills (28), and in urinary output of sodium, potassium and chloride by other workers (29), during acute CO_2 inhalation in man. Similarly, in our Experiment 6, with NaCl substituted for the small pre-medication dose of $NaHCO_3$ (and a control urine pH of 5.9) there was no detectable change in UV_{H^+}. In spite of the acid control urines, it seems probable that a renal tubular response could have been found in the experiments of Longson and Mills (28) had changes in filtered load and reabsorption of bi-

[7] The use of the index proposed by these authors involves the assumption that the basic tubular process of H^+ secretion is involved in the entire quantity of HCO_3^- reabsorbed. This view is presently held by most workers, as they point out, but it has also been recently challenged (16).

[8] For the bicarbonate administration studies renal adjustment was calculated by an alternative approximation rather than by ΔUV_{H^+} as defined in this paper. From the laws of buffer action and electroneutrality it follows that changes in UV_{H^+} must be accompanied by an equal change in excretion of fixed cation minus fixed anion of opposite algebraic sign. Under many circumstances, including our own experimental conditions, a fairly satisfactory approximation of ΔUV_{H^+} can be derived from changes in excretion rates of the major fixed ions of urine, as $Na^+ + K^+ - Cl^-$. Expression of the urinary adjustment in these alternative terms directs attention to the effect on body fluid content of the major fixed ions, which may be convenient in consideration of acid-base changes there. Use of this approximation during hyperventilation indicates an average change of $+222$ μEq. per min., surprisingly close to the ΔUV_{H^+} of -218 μEq. per min. by the other method.

carbonate been determined rather than in excretion alone. Data of Denton, Maxwell, McDonald, Munro, and Williams (30) during CO_2 inhalation in sheep indicate a marked rise in HCO_3^- reabsorption, although, like Longson and Mills, they observed no change in rate of urinary excretion of HCO_3^-. In contrast to these negative urinary results other workers have found characteristic changes in urinary acid-base factors in acute respiratory acidosis in dogs (15, 17, 31).[9] Where given, control urinary pH values in these experiments were close to 7.0 or above. We observed significant urinary changes in the five subjects who were given the preceding oral dose of $NaHCO_3$, and in whom the control urine pH was also approximately neutral.

Other factors influence the characteristic renal response observed during respiratory acid-base disturbances. For example, the typical urinary response to respiratory alkalosis is lacking in subjects who are in a state of NaCl depletion (18, 20). It is to be expected that pre-existing disturbances of the circulation or fluid and electrolyte balance, endocrine factors, and kidney disease may alter the characteristic changes that have been defined.

In more prolonged respiratory disturbances (for example of four or five days' duration) the ability of the kidney to compensate appears increased over that seen initially (32). Ammonium excretion is likely to constitute a larger fraction of the renal response to more prolonged acidosis. Although NH_4^+ output begins to rise within a few minutes of onset of acidosis, it does not approach maximal rates for some hours or days (33).

The urinary findings in different phases of respiratory acid-base disturbances

It must be emphasized that the characteristic combination of urinary changes described above is typical only of the "displacement" phase of the respiratory disturbance. While changes in urinary rates of H^+ excretion persist they result in a progressive increase in the degree of metabolic acidosis or alkalosis which compensates in part for the respiratory disturbance. If the abnormal level of PCO_2 persists for periods much longer than the duration of the present experiments, eventually a point will be reached where the secondary change in extracellular buffer base and other chemical changes have restored the pH to a level of stability in or closer to the normal range. In the ensuing "steady state" phase of the disturbance renal compensation ceases, in the sense that no further progressive change is produced in buffer base of body fluids. Output of H^+ then reflects the dietary intake and other physiological processes, as it does in a state of normal respiration and PCO_2. Finally, during the "recovery" phase of a chronic respiratory acid-base disturbance, when PCO_2 is returning toward normal, renal compensation serves to restore the extracellular buffer base to normal. Changes are therefore the precise opposite of those described for the displacement phase: increase in UV_{H^+} in chronic respiratory alkalosis, and decrease in chronic respiratory acidosis. Thus in chronic respiratory disturbances a temporary increase in the respiratory difficulty (equivalent to an additional "displacement" phase) will be accompanied by reappearance of characteristic urinary changes; a temporary decrease in the respiratory difficulty (equivalent to a partial "recovery" phase) will cause the opposite type of urinary changes. Such a sequence of events is quite otherwise to the situation found in metabolic acid-base disturbances. In a complete study of the displacement, stabilization, and recovery phases of ammonium chloride acidosis, Sartorius, Roemmelt, and Pitts (33) have shown that the fundamental urinary response is one of increased UV_{H^+} in all of these phases, despite serial differences in individual constituents that are also of importance. The same holds true for the opposite changes in metabolic alkalosis produced by electrolyte addition (25, 26). In acute respiratory experiments of brief duration, such as those reported here, there is no stabilization, and recovery is marked simply by a return of the urinary acid-base output toward control levels.[10] Superimposed on this return,

[9] A change is evident in the urinary data of Fuller and MacLeod (15), although their calculation of "total H^+ secretion" did not change significantly because of a fall in glomerular filtration rate and therefore in bicarbonate reabsorption. Their method of calculation showed that 90 to 98 per cent of "total H^+ secretion" was accounted for by bicarbonate reabsorption in respiratory disturbances.

[10] Fuller and MacLeod (15) reported that urinary effects of respiratory acidosis were rapidly reversible,

however, are the effects of other factors, such as diurnal fluctuations (34), the wearing off of the slight metabolic alkalosis previously induced in the subjects exposed to CO_2 inhalation, persistence of slight differences in respiration, and late effects of the imposed diuresis.

Once the "balance" between the existing respiratory disturbance and the compensatory metabolic acidosis or alkalosis (produced by the kidney) has been reached, there similarly may be nothing distinctive about the urinary excretion for individual ions except as related to the accompanying composition of plasma. For example, in one of our unpublished cases of chronic CO_2 retention with a plasma PCO_2 of 120 mm. Hg, urinary chloride excretion exceeded 40 mEq. per day, corresponding approximately to the intake, despite an extremely low plasma chloride concentration of 74 mEq. per liter. It is not surprising that the observer may erroneously conclude that renal compensations are unimportant in such disturbances if he directs his attention to the urinary composition alone. Determination of clearance rates or reabsorptive rates for acid-base factors will, however, disclose the presence of renal response.

Regulation of renal bicarbonate excretion

Change in the rate of excretion of bicarbonate was the principal anionic response of the kidney to the respiratory disturbances. In a preliminary report of the present work (3) it was stated that bicarbonate *excretion* rate correlated better with plasma pH than with other plasma factors such as PCO_2 or HCO_3^- concentration. About the same time Brazeau and Gilman (35), Dorman, Sullivan, and Pitts (36), and Relman, Etsten, and Schwartz (37) each reported studies showing that the *reabsorption* of bicarbonate was more closely related to the PCO_2 of the blood than to pH or concentration of bicarbonate. Several recent references misconstrue our data and interpretations as in disagreement with the above groups. This confusion has resulted from the failure to note the terms in which the data are reported. Examination of the directional changes given in Figure 4 will disclose that actually no disagreement exists. In our consideration of the over-all effect of the renal compensation on the body we stressed output (excretion). The figure illustrates that HCO_3^- excretion is decreased in respiratory acidosis and increased in both respiratory and metabolic alkalosis. Since both plasma PCO_2 and plasma HCO_3^- concentration move in the opposite directions in respiratory alkalosis and metabolic alkalosis, it is obvious that HCO_3^- *excretion* is better correlated with plasma pH. The three groups of investigators mentioned above, however, were interested primarily in the renal component due to active tubular processes. In studying the regulation of these processes they therefore logically stressed bicarbonate *reabsorption*. Figure 4 shows that the rate of HCO_3^- reabsorption is decreased in respiratory alkalosis but increased in metabolic alkalosis (electrolyte addition). Obviously, then, reabsorption does not correlate well with plasma pH but could be correlated (in direction of change) with either plasma bicarbonate concentration or PCO_2.[11] In experiments carefully designed to test this point the groups mentioned above found in dogs that PCO_2 was the more closely related. If our human data are used for calculation of correlation coefficients it is found that reabsorptive rate does correlate somewhat better with PCO_2 than with plasma bicarbonate concentration. Thus, it should be apparent that conclusions about changes in bicarbonate *excretion* cannot be considered as simply the opposite of changes in *reabsorption*. If the filtered load changes sufficiently, rates of reabsorption and excretion may change simultaneously in the same direction, as happened in the bicarbonate administration experiments.

Renal excretion of potassium

The predominance of changes in potassium excretion rate over those of sodium is a striking result since the sodium greatly exceeds potassium in the glomerular filtrate. At the end of either hyperventilation or CO_2 inhalation the significance of change in potassium excretion compared to con-

while hyperventilation produced lasting depression of titratable acid excretion and increase in bicarbonate excretion. These findings in anesthetized dog experiments differ from the return to control values after hyperventilation noted in humans in our studies and those of Stanbury and Thomson (20).

[11] Several workers have advanced the suggestion that this effect may be mediated through changes in pH within the tubule cells (38, 39).

trol rate clearly exceeded that of the corresponding changes in sodium excretion. Indeed in the hyperventilation experiments even the absolute magnitude of increased excretion was much greater ($K^+ + 183$ μEq. per min., $Na^+ + 70$ μEq. per min.). Since potassium is the predominant intracellular cation one might suggest that the release of potassium from body cells as a compensatory mechanism to hyperventilation is involved. A close relation between potassium and buffering action of intracellular fluid has been prominently considered since the work of Darrow and his associates (40, 41) on certain potassium deficiency states. The calculated "cellular exchanges" of potassium in our experiments (1) would affect extracellular concentration in the same direction as do the changes in urinary excretion of potassium. Thus they are in the direction opposite to that which would be required to explain the urinary findings through a change in plasma potassium concentration and secondarily in renal load. The change in potassium excretion under these circumstances might result from a reciprocal relationship between the tubular secretions of hydrogen ion and potassium as suggested by Berliner, Kennedy, and Orloff (42).

Sodium and chloride excretion.

In extracellular fluid, changes in buffer base are approximately equal to changes in $Na^+ - Cl^-$. Yet during the first half hour of respiratory acid-base disturbances those changes that did occur in sodium and chloride excretion tended to be in the same direction. Therefore urinary $Na^+ - Cl^-$ excretion showed little variation and change in fixed ions accompanying ΔUV_{H^+} was associated predominantly with K^+. The alterations in sodium and chloride excretion were not directly correlated with the continuation and withdrawal of the experimental stimuli, and were not necessarily opposite in direction in the acidosis from the alkalosis experiments. Our data therefore do not support the suggestion of Stanbury and Thomson (20) that a fall in chloride output following acute hyperventilation may represent a separate acid-base mechanism favoring conservation of "fixed acid" (Cl^-). Since these parallel changes of sodium and chloride excretion are not concerned with preserving acid-base homeostasis, some other physiological process must be involved. This might concern mechanisms of electrolyte conservation, hormonal factors, or renal hemodynamic changes. Renal plasma flow, but not glomerular filtration rate, showed a positive correlation with sodium and chloride excretion in the hyperventilation studies, while neither showed statistically significant changes with CO_2 inhalation.

Excretion of other ions

Phosphate excretion changes in two ways: as the rate of total phosphate output (in μM per min.) and as the proportion as $HPO_4^=$ or as $H_2PO_4^-$. Changes in the buffer role of phosphate as a transporter of H^+ are included in the titratable acidity, of which phosphate is an important part. This may be expressed quantitatively by the difference between the excretion rate of phosphate in μEq. per min. at the observed pH and the calculated rate in μEq. per min. that would occur if the same number of μM were excreted at a pH of 7.4. An increase in μM per min. phosphate excretion is regularly observed in respiratory acidosis (17, 30, Table I B) and a decrease in respiratory alkalosis (17, 19, 20, Table I A), but these changes are small relative to the other changes. Calculated *undetermined anion* excretion showed only very small changes in both types of experiments. It is therefore unlikely that changes in *excretion* of sulfate, lactate or organic acids are quantitatively important compared to the other changes reported.

SUMMARY

Acute respiratory alkalosis by voluntary hyperventilation for approximately thirty minutes, or acidosis by CO_2 inhalation for a similar period, were induced in normal human subjects. The urinary excretion of water and electrolytes and the acid-base pattern of the urine were observed in multiple clearance periods before, during and after the respiratory stimuli.

In *respiratory alkalosis* the kidney responded promptly by retaining hydrogen ion compared to control excretion, measured principally as an increase in output of bicarbonate with potassium. Urinary pH rose and titratable acidity, ammonium ion, and phosphate excretion fell. The potassium effect appeared to be due to renal regulation rather than secondary to systemic intracellular adjust-

ments. Chloride excretion tended to vary with that of sodium, increasing slightly during hyperventilation and then falling far below the control level.

Changes observed during *respiratory acidosis* were, for most variables, opposite in direction to those noted during hyperventilation. They were smaller in magnitude since the experimental acute respiratory acidosis by CO_2 inhalation was a milder acid-base disturbance than hyperventilation as indicated by degree of plasma changes and estimation of total extracellular acid-base disturbance. Changes in excretion of sodium and chloride were not as definite as those in other factors and were not always opposite in direction in the two types of experiment. At least part of this change (when sodium and chloride change in the *same* direction) appears not immediately concerned with acid-base homeostasis. If the urine is already acid the typical urinary changes may not be evident during acute CO_2 inhalation.

Renal mechanisms account for only a small part of the adjustments observed in experiments of short duration, but become more important with prolonged stimuli. The rate of renal acid-base compensation is considerably greater in acute extracellular alkalosis of similar magnitude induced by sodium bicarbonate administration. The urinary patterns described are typical of the "displacement" phase of respiratory disturbances. With chronic respiratory disturbances a stabilized situation may be reached in which changes are not evident in rates of urinary output of hydrogen ion except as compared to abnormalities of the acid-base composition of plasma; if "recovery" then occurs the direction of hydrogen ion excretion typical of the "displacement" phase is reversed.

The data are compatible with the finding of others that the tubular *reabsorption* of HCO_3^- is better correlated with plasma PCO_2 than with other extracellular acid-base factors. Changes in *excretion* of HCO_3^-, however, which determine the effect on the body, correlate with plasma pH and not with plasma PCO_2, if one considers both respiratory and metabolic disturbances.

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BILE ACID CONTENT OF HUMAN SERUM. I. SERUM BILE ACIDS IN PATIENTS WITH HEPATIC DISEASE [1]

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Lack of specific analytic methods has hitherto prevented exact studies of the effect of liver disease upon the concentration of bile acids in the serum. Until recently it has not been possible to measure the serum concentration of bile acids other than cholic acid. Various modifications of the Pettenkofer reaction have been employed to determine the serum concentration of cholic acid. Reports of "normal" serum cholic acid concentrations of 0.0 mg. per cent (1), 0.4 mg. per cent (2), 0.6 to 2.2 mg. per cent (3), and 3 to 6 mg. per cent (4) reflect the non-specific nature of this reaction. Several observers have agreed, however, that an accumulation of cholic acid occurs in the serum of patients with obstructive jaundice, elevations to 9 mg. per cent having been recorded (5). In acute hepatitis, serum cholic acid was less regularly elevated (5). In patients with portal cirrhosis, serum cholic acid as studied by Snell (6) and by Sherlock and Walshe (5) was generally not elevated.

The introduction of improved methods of separating and identifying the various bile acids (7–9) has stimulated a re-examination of this subject. A preliminary report from this laboratory (10) indicates that both dihydroxy and trihydroxy bile acids, largely in conjugated form, accumulate in the serum and are excreted in the urine of patients with obstructive jaundice. Only dihydroxy bile acid has been found in the serum of patients with Laennec's cirrhosis and no bile acid can be detected in the urine of these subjects.

The binding of bile acids by serum albumin has also been described (10). Carey (11) has reported differences in the ratio of dihydroxy and trihydroxy bile acids in the sera of patients with obstructive jaundice and hepatocellular disease.

The present report is concerned with: a) the bile acid content of the serum and urine in a selected series of 40 patients, 30 of whom had hepatic disease; b) correlations between serum bile acid values and certain other manifestations of the hepatic disorder; c) a consideration of the mechanisms responsible for certain differences of serum bile acid pattern in various types of hepatic disease.

CLINICAL MATERIAL

The subjects in this study were separated into five groups.

Group I: Ten persons without liver disease. Of these subjects, two were healthy volunteers; five had hypertensive vascular disease without cardiac or renal decompensation; one had idiopathic hyperlipemia and arteriosclerotic cardiovascular disease; one had diabetes mellitus with Kimmelstiel-Wilson syndrome; and one had bronchial asthma.

Group II: Thirteen patients under treatment for Laennec's cirrhosis. All reported a history of chronic alcoholism and inadequate diet, and presented the characteristic physical findings of Laennec's cirrhosis including ascites. There was considerable variation in the severity and stage of the disease.

Group III: Ten cases of hepatic disease associated with obstructive jaundice. Diagnoses in this group were as follows: post-operative bile duct stricture (one patient); congenital atresia of the bile duct (one patient); primary cholangitis (three patients); xanthomatous biliary cirrhosis (two patients); hepatic sarcoidosis (one patient); and two cases of chronic hepatitis with a pronounced component of biliary obstruction. All diagnoses were proved by operation, biopsy, or necropsy with the exception of patient No. 26 (Table I) in whom biopsy was not performed. All patients in this group had a persistent elevation of serum alkaline phosphatase and serum cholesterol.

Group IV: Five cases of chronic hepatitis, without evidence of biliary obstruction. In all these patients, the features of the chronic hepatic disease included jaundice present for periods of 1 to 22 months; positive cephalin flocculation or zinc turbidity test; alkaline phosphatase

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TABLE I—*Bile acid content of serum and urine*

Case No., Age, Sex	Clinical diagnosis	Bilirubin (mg. %)	Ceph. flocc. at 24 hrs.	Zinc turb.	A/G (gm. %)	Alk. phos. units* (%)	Cholesterol tot./est. (mg. %)	Serum bile acids (mg. %) DBA†	Serum bile acids (mg. %) TBA†	Serum bile acid ratio TBA/DBA	Urinary bile acids (mg./24 hrs.) DBA	Urinary bile acids (mg./24 hrs.) TBA
Group I												
1. 62, F.	Hypertensive cardiovascular disease							0	0			
2. 50, F.	Hypertensive cardiovascular disease							0	0			
3. 55, F.	Hypertensive cardiovascular disease							0	0		0	0
4. 48, F.	Hypertensive cardiovascular disease							0	0			
5. 52, M.	Hypertensive cardiovascular disease							0	0		0	0
6. 58, M.	Hyperlipemia; arteriosclerotic C.V. disease							0	0		0	0
7. 23, F.	Diabetes, Kimmelstiel-Wilson							0	0		0	0
8. 42, F.	Bronchial asthma							0	0		0	0
9. 29, M.	Normal							0	0		0	0
10. 28, F.	Normal							0	0			
Group II												
11. 64, M.	Laennec's cirrhosis	0.9	1+		3.9/4.2	2.3 B.U.	172/122	1.4	0	0	0	0
12. 39, M.	Laennec's cirrhosis	0.3	0		1.4/4.1	3.5 B.U.	258/178	0.6	0	0		
13. 49, F.	Laennec's cirrhosis	4.5	4+		3.0/5.6	7.9 B.U.	221/90	5.0	0.6	0.12	0	0
14. 56, M.	Laennec's cirrhosis	8.4	3+		2.9/3.0		189/82	2.2	0	0		
15. 53, F.	Laennec's cirrhosis	2.4	4+		4.1/2.2	2.9 B.U.	218/126	2.5	0.4	0.16	0	0
16. 50, M.	Laennec's cirrhosis	0.8	2+		3.1/2.0	2.6 B.U.	119/82	1.0	0	0	0	0
17. 54, F.	Laennec's cirrhosis	0.5	0		3.1/3.1			1.3	0	0	0	0
18. 41, F.	Laennec's cirrhosis	6.8	4+		2.7/2.9	21.4 K.A.	136/56	3.0	0	0	4.0	0
19. 56, F.	Laennec's cirrhosis	7.6	3+		4.0/5.2	1.8 B.U.	244/104	1.0	0	0	7.3	0
20. 56, M.	Laennec's cirrhosis	0.6	0		4.3/1.1	7.3 B.U.	168/121	0.6	0	0	0	0
21. 54, F.	Laennec's cirrhosis	8.9	4+		2.9/2.3	1.8 B.U.	135/92	0	0	0		
22. 49, M.	Laennec's cirrhosis	1.5	2+		2.8/2.8	3.1 B.U.	151/103	1.4	0	0		
23. 67, M.	Laennec's cirrhosis	0.4	0		3.1/3.0	1.8 B.U.	177/125	0.7	0	0		
Group III												
24. 20 mo. F.	Chronic hepatitis with biliary obstruction	2.0		6.4	4.5/1.8	16.8 B.U.	550/290	1.0	2.0	2.0	0	0
25. 15 mo. F.	Chronic hepatitis with biliary obstruction	10.1		6.8	3.9/2.3	34.3 B.U.	1027/802	2.4	11.1	4.6	9.4	4.5
26. 4 yr. M.	Xanthomatous biliary cirrhosis	11.1		26.	3.7/3.0	29.2 B.U.	706/280	3.4	14.8	4.4	17.4	120.8
27. 18 mo. F.	Congenital atresia of bile ducts	13.5	2+	18.7	3.7/3.2	19.3 B.U.	743/363	5.4	5.0	0.93		
28. 49, F.	Xanthomatous biliary cirrhosis	21.2	3+		4.2/5.1	55.8 K.A.	850/148	5.6	11.0	2.0	28.4	25.0
29. 19, M.	Hepatic sarcoidosis	3.3	0		4.5/2.5	23.6 B.U.	270/181	2.0	2.8	1.4	9.0	26.0
30. 32, F.	Common duct stricture	10.3	1+		3.6/3.2	36.6 K.A.	264/72	3.2	3.2	1.0		
31. 55, M.	Primary cholangitis	8.1	0		4.0/3.1	7.3 B.U.	296/132	0.5	1.5	3.0		
32. 55, M.	Primary cholangitis	3.8	0		4.8/2.1	8.5 B.U.	406/252	4.3	11.4	2.7	25.6	25.0
33. 46, M.	Primary cholangitis	14.1	0			89.1 K.A.		1.3	3.5	2.7		
Group IV												
34. 4 mo. M.	Chronic hepatitis	16.5	0	9.2	4.6/2.3	24.2	248/—	3.9	1.2	0.31	2.0	2.8
35. 6 mo. F.	Chronic hepatitis	8.2	0	4.2	4.6/2.4		264/152	2.8	1.3	0.46	3.1	4.3
36. 3 yr. F.	Chronic hepatitis	18.8	0	23.4	3.7/3.9		322/140	6.4	3.4	0.53		
37. 20, M.	Chronic hepatitis	7.25	2+		2.4/4.2	9.8 B.U.	129/54	3.7	1.4	0.38	13.0	17.1
38. 69, M.	Chronic hepatitis	10.9	3+		2.6/4.8	7.8 B.U.	99/29	3.5	1.4	0.40	12.4	0
Group V												
39. 25, F.	Acute infectious hepatitis	24.8	4+		5.5/1.4	2.2 B.U.	264/145	4.1	4.4	1.1	4.6	9.2
40. 64, M.	Acute hepatitis due to Androgen administration	4.4	2+		4.5/2.6	35.2 K.A.	167/117	1.2	1.4	1.2		

* B.U., Bodansky units; K.A., King-Armstrong units. † DBA, dihydroxy bile acid; TBA, trihydroxy bile acid.

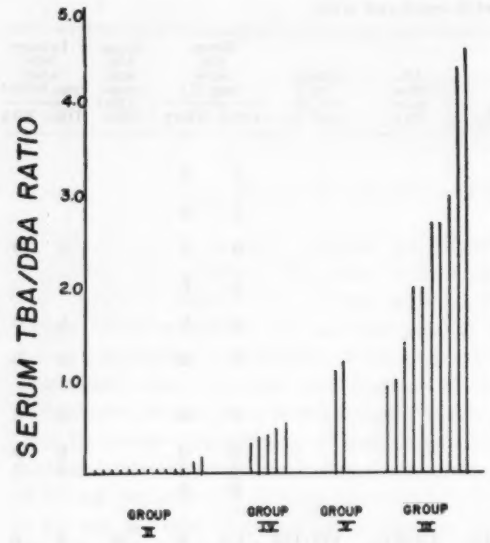

FIG. 1. SERUM TBA/DBA RATIO AMONG THE VARIOUS GROUPS OF PATIENTS WITH HEPATIC DISEASE

and serum cholesterol within normal limits;[8] and negative history for alcoholism, dietary imbalance, or hepatotoxic agents. Liver biopsies substantiating the diagnosis of chronic hepatitis were performed on Cases Nos. 34 and 35 (Table I); biopsy was not done on the other three patients in this group.

Group V: Two cases of acute hepatitis. One patient was a 25-year-old woman with acute hepatitis, presumably of viral etiology, who made an uncomplicated recovery. Liver biopsy was not performed in this patient. The second subject was a 64-year-old man who became jaundiced two months after commencement of the administration of a testosterone analogue. Liver biopsy indicated acute liver cell injury. Following discontinuance of the drug, the findings of liver damage rapidly disappeared.

METHODS

Analysis was carried out on a 4 to 15-ml. sample of fresh serum. Bile acid concentration was found to be unaffected by preservation of serum for several months at $-20°$ C. An alcoholic extract of the serum sample was made as described by Josephson (3). The extract, after removal of the barium salts and neutralization, was concentrated to a volume of about 30 ml. on the steam bath. An equal volume of H_2O was added, the pH was adjusted to 9, and the solution was extracted three times with petroleum ether (B.P. 68° C) to remove triglycerides and cholesterol. The aqueous solution was neutralized and evaporated to dryness. The bile acid content of this residue was determined by one of two procedures:

In one procedure, used for the determination of the total bile acid content, the residue was dissolved in 20 ml. 5 per cent NaOH and hydrolyzed by heating for 3 hours in an autoclave at 15 lbs. pressure. After cooling, the aqueous solution was made acid to Congo Red with concentrated HCl and extracted five times with equal volumes of ethyl ether. After being dried over Na_2SO_4 the ethereal solution was taken to dryness. The residual material was fractionated by partition chromatography according to the method of Mosbach, Zomzely, and Kendall (7). The first fraction (100 per cent petroleum ether) was discarded. The second and third fractions (60 per cent petroleum ether, 40 per cent isopropyl ether; and 40 per cent petroleum ether, 60 per cent isopropyl ether, respectively) were taken to dryness and acetic acid was removed by repeated evaporation after the addition of benzene. Aliquots from fraction 2 were analyzed spectrophotometrically for total dihydroxy bile acid and for chenodeoxycholic acid (7, 12); and from fraction 3 for trihydroxy bile acid.

In the other procedure, used when information concerning the conjugation of the bile acids was desired, the residue was taken up in 50 ml. of ethanol and the insoluble portion removed by filtering. Aliquots of the ethanolic solution, after acidification, were fractionated by the reverse phase chromatographic systems of Norman (13). Appropriate fractions from the column were hydrolyzed, acidified to Congo Red, and free bile acid recovered by ether extraction. Di- and tri-hydroxy bile acids were then determined spectrophotometrically.

The bile acid content of a 50-ml. aliquot of urine was determined as follows. After the addition of 2.5 gm. of NaOH, hydrolysis was carried out in the autoclave at 15 lbs. for 3 hours. The solution was treated thereafter exactly as described for the hydrolysate of the material derived from serum.

RESULTS

Serum and urine bile acid content

Table I gives data on the bile acid content of the serum and urine of the 40 subjects, together with other laboratory findings pertinent to the hepatic disease. Figure 1 illustrates the distribution of the serum trihydroxy/dihydroxy bile acid ratios among the four groups of patients with hepatic disease.

No bile acid could be detected in the serum or urine of any of the subjects without hepatic disease (Group I).

In 12 of 13 patients with Laennec's cirrhosis (Group II), the serum contained dihydroxy bile acid (DBA) in amounts of 0.6 to 5.0 mg. per cent. Trihydroxy bile acid (TBA) was detectable in only 2 of these cases, at a level of 0.4 and 0.6 mg.

[8] Case No. 38 (Table I) in this group exhibited, during certain periods of his illness, a borderline elevation of the serum cholesterol and alkaline phosphatase.

TABLE II

Serial bile acid determinations

Case No.	Date	Serum DBA mg. %	Serum TBA mg. %	Bilirubin mg. %	Comment
13	7/28/55	6.2	0	5.5	Laennec's cirrhosis; status quo
	8/8/55	7.2	0	5.5	
	9/19/55	5.0	0.6	4.5	
	9/28/55	5.4	0	4.7	
	10/11/55	5.9	0	4.7	
15	1/9/56	2.5	0.4	4.3	Laennec's cirrhosis; gradually receding jaundice
	2/15/56	1.7	0	2.4	
	3/12/56	1.7	0	2.1	
38	5/1/56	3.0	2.7	10.2	Chronic hepatitis in exacerbation; death on 5/26/56
	5/4/56	3.5	1.4	10.9	
	5/10/56	5.0	2.9	11.6	
40	8/26/55	4.1	4.4	24.8	Acute viral hepatitis; complete recovery
	9/7/55	2.5	3.6	11.0	
	9/21/55	1.3	0	3.2	
	9/30/55	0	0	0.8	

per cent. The one subject (Case No. 21) in whom dihydroxy bile acid was not detectable in the serum was in the terminal stage of hepatic coma at the time of the analysis, following hemorrhage from esophageal varices. Urinary bile acid was determined in 8 individuals in this group. In 2 cases dihydroxy bile acid was present in the amounts of 4.0 mg., and 7.3 mg. per 24 hrs.

In all 10 cases of obstructive jaundice (Group III), the serum contained both di-and trihydroxy bile acid. TBA concentration exceeded that of DBA in 8 cases. The ratio of TBA to DBA serum concentration varied from 0.9 to 4.6. Five of the 6 patients whose urine was analyzed, excreted both types of bile acids in the urine. The 24-hr. bile acid excretion in the adult patients varied from 9.0 mg. to 28.4 mg. for DBA; from 25.0 mg. to 26.0 mg. for TBA.

The serum of all patients with chronic hepatitis in Group IV contained both di- and trihydroxy bile acid. Dihydroxy bile acid concentration exceeded that of trihydroxy bile acid, the TBA/DBA ratio varying from 0.3 to 0.5. Bile acids were detected in the urine in all cases studied.

In one case of acute viral hepatitis (Group V), on the 20th day of the illness the serum DBA was 4.1 mg. per cent and the TBA 4.4 mg. per cent. In a case of acute hepatic injury due to drug toxicity, serum DBA was 1.2 mg. per cent and serum TBA 1.4 mg. per cent.

Serial determinations of serum bile acids

In 4 patients the bile acid concentrations were determined repeatedly during the hospital course (Table II). There was little variation in the concentration of serum DBA in 2 patients with Laennec's cirrhosis, who exhibited little change in clinical condition during the intervals encompassed by these determinations.

On the other hand, in a man with chronic hepatitis, the serum bile acids were determined three times during an exacerbation of his disease. The serum DBA level rose steadily while the patient's condition worsened, with progressive lethargy and the onset of neurological symptoms which culminated in coma and death.

The serum of a 25-year-old woman with acute viral hepatitis was analyzed three times for bile acids during the favorable course of her illness. The decrease in serum bile acids paralleled the rapid clinical improvement.

Observations on the structure of the serum bile acids

Since the foregoing analyses of hydrolyzed extracts of serum provided no information concerning the state of conjugation, or mode of transport, of the serum bile acids; and since these details are crucial in the consideration of the mechanisms which underly the presence of bile acids in the

TABLE III

Conjugated bile acid content of serum

Case No.	Serum DBA			Serum TBA		
	Total (*mg.* %)	% as taurine conjugate	% as glycine conjugate	Total (*mg.* %)	% as taurine conjugate	% as glycine conjugate
28				11.0	34	45
33				3.5	38	42
13	4.7	0	18			
14	2.1	0	12			
15	2.3	0	35			

serum and urine of patients with hepatic disease, the following studies were performed:

a) *State of conjugation.* Preliminary studies, employing reverse phase partition chromatography of unhydrolyzed serum extracts, have been carried out to determine the state of conjugation of the serum bile acids (Table III). In 2 patients with biliary obstruction 34 to 38 per cent of the serum TBA was present as the taurine conjugate, and 42 to 45 per cent as the glycine conjugate. Three serum samples from patients with Laennec's cirrhosis were studied prior to hydrolysis. No taurine conjugated DBA was detectable. Twelve, 18 and 35 per cent, respectively, of the DBA in each of these samples exhibited the mobility of the glycine conjugate on the column. Further studies are in progress aimed at identifying the remaining fractions of the total DBA content of the serum in Laennec's cirrhosis.

b) *Nature of the dihydroxy bile acid.* The chenodeoxycholic acid (3, 7-dihydroxy cholanic acid) content of serum has been determined in several patients, by means of the specific colorimetric reaction of Isaksson (12) (Table IV). Chenodeoxycholic acid was found to constitute 56 to 100 per cent of the serum DBA in these patients.

Further characterization of the trihydroxy bile acid of serum has not yet been carried out. The ultraviolet absorption spectrum after heating with 65 per cent sulfuric acid, and the chromatographic behavior of this substance, are compatible with the 3, 7, 12-trihydroxycholanic acid structure of cholic acid, the only trihydroxy bile acid which has been identified in human bile.

c) *Interaction of bile acids with serum proteins.* Analysis of dialysates prepared from serum samples with a considerable bile acid content indicated that only a small proportion of the trihydroxy acid, and no detectable fraction of the dihydroxy bile acid, was diffusible (14). Since the dialysis membrane is freely permeable to the various bile acids, this observation suggested an extensive binding of the bile acids by serum proteins. In a series of dialysis-equilibrium experiments, reported in another paper (15), the binding activity was found to reside largely in the albumin fraction.

DISCUSSION

Serum bile acid ratios in different types of hepatic disorder

In patients with liver disease the nature of the hepatic disorder has a profound effect upon the relative amounts of tri- and dihydroxy bile acids present in the serum and upon their state of conjugation.

Figure 1 shows the ratios found between tri- and dihydroxy bile acids in patients with different types of hepatic disease. In Laennec's cirrhosis (Group II) the serum bile acid is almost entirely of the dihydroxy type, trihydroxy bile acid being found in small amounts in only 2 of 13 patients studied. In biliary obstruction (Group III), the concentration of the trihydroxy acid is from 1 to 4 times that of the dihydroxy bile acid. Serum bile acid ratios intermediate between those of Group II and Group III are found in the patients with hepatitis (Groups IV and V). No values are given for normal individuals since their serum levels are too low to measure by our methods. In normal human gall bladder bile, the ratio between the tri- and dihydroxy bile acids averages about 0.75 (16).

In obstructive liver disease, the serum bile acids are largely conjugated with taurine and glycine in roughly equivalent amounts (Table III) and in this respect resemble the bile acids found in nor-

mal gall bladder bile. In patients with Laennec's cirrhosis, less than 35 per cent of the serum bile acid is conjugated and only the glycine conjugate is found.

A clear understanding of the pathologic mechanisms which underlie these findings must await more detailed knowledge of the metabolic origin of the cholanic acids than is available at present. Although Bloch, Berg, and Rittenberg (17), Siperstein and Chaikoff (18), and Bergström (19) have demonstrated that the bile acids are end-products of the degradation of cholesterol, the details of the metabolic pathways involved are not yet defined. Certain observations, however, may help understand the reasons why the bile acids present in the serum of patients with biliary obstruction are different from those present in patients with Laennec's cirrhosis and why, in both diseases, the serum bile acids differ from those normally excreted in the bile. Bergström and Gloor (20, 21) have demonstrated the presence of an enzyme system in the liver cells of rats that converts deoxycholic (dihydroxy) acid into cholic (trihydroxy) acid, and of another enzyme that conjugates the free bile acids with glycine and taurine. This conjugating enzyme has been shown (21) to be present in normal human liver cells and it is probable that the hydroxylating enzyme also is present. If it is assumed that both of these enzyme systems are present and active in the liver cells of patients with biliary obstruction, the bile acids regurgitated into the serum should be completely conjugated. The percentage of trihydroxy acid in the serum of patients with biliary obstruction is considerably higher than in normal human bile (16); this difference might possibly arise from the longer retention of the bile acids within the body, thus permitting a more protracted action of the hydroxylating enzyme system.

The nature of the bile acids found in the serum of patients with Laennec's cirrhosis suggests a reduction in the activity of both the conjugating and hydroxylating enzyme systems in this disease. The failure to conjugate bile acids is paralleled by a similar failure to conjugate bilirubin. Schmid has reported (22) that bilirubin conjugated with glucuronic acid predominates in the serum of patients with regurgitation jaundice, while in retention jaundice most of the serum pigment is in the form of free bilirubin. The accumulation of unconjugated bile acids in the serum of these patients, in spite of unobstructed bile flow, suggests that the liver cells are unable to excrete unconjugated bile acids into the bile ducts. The presence of these acids in the serum of patients with Laennec's cirrhosis may be due largely to the failure of the conjugating enzyme system.

TABLE IV

Chenodeoxycholic acid content of serum

Case No.	Serum DBA mg. %	Serum CDCA* mg. %	CDCA as % of DBA
13	5.0	3.7	74
15	2.5	1.6	65
19	1.0	0.7	70
25	2.4	2.4	100
26	3.4	2.6	77
31	0.5	0.4	76
34	6.4	4.6	72
36	3.5	2.0	56
38	3.9	2.8	73

* CDCA, chenodeoxycholic acid.

A variable degree of liver cell damage is undoubtedly present in most patients with obstructive jaundice and may perhaps be responsible for the variation in the ratio of tri- and dihydroxy bile acids found in these patients. The lowest ratios in this group were found in two patients (Nos. 27 and 30) with longstanding complete obstruction and marked impairment of liver cell function.

No information has been obtained in this study concerning the conjugation of serum bile acids in acute and chronic hepatitis. The ratios between the tri- and dihydroxy acids are intermediate between those found in Groups II and III. This suggests that cellular damage affecting the conjugating and hydroxylating enzyme systems is superimposed upon biliary obstruction in these patients.

Serum bile acids in relation to other manifestations of hepatic dysfunction

The serum bile acid concentration of the patients was compared with other laboratory findings indicative of the presence of hepatic disease (Table I).

No consistent relationship could be demonstrated between the level of bile acids and ab-

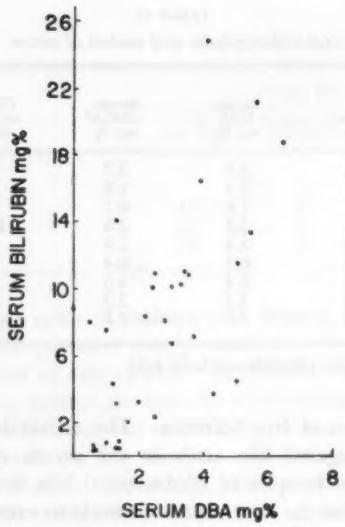

FIG. 2. RELATIONSHIP OF SERUM DIHYDROXY BILE ACID CONCENTRATION TO SERUM BILIRUBIN CONCENTRATION IN PATIENTS WITH HEPATIC DISEASE

normal values for cephalin flocculation or albumin-globulin ratio. The patients with appreciable serum levels of trihydroxy acids tended to have high values for serum alkaline phosphatase. These patients also exhibited high serum cholesterol levels. In all types of liver disease studied, high serum levels of bile acid tended to be associated with high values for serum bilirubin (Figure 2). In our series the correlation between these values was better for the patients with Laennec's cirrhosis, where practically all the serum bile acid was of the dihydroxy type, than for the patients with biliary obstruction.

In the patients with Laennec's cirrhosis, the serum DBA level did not reflect the over-all severity of the disease. Several patients with Laennec's cirrhosis advanced enough to produce neurological symptoms (Cases Nos. 11, 17), severe portal hypertension (Cases Nos. 16, 17, 20, 21) or marked hypoalbuminemia (Cases Nos. 12, 21, 22), exhibited relatively low serum DBA. Although serum DBA concentration showed little fluctuation in the individual cirrhotic patient (Table II), it was highly variable from one patient to another. Serum DBA concentration undoubtedly is determined by the interplay of several factors, including the rate of conversion of cholesterol to bile acid and the concentration of transporting plasma proteins.

Excretion of bile acids in the urine

Bile acids were found in the urine of all patients with obstructive jaundice having appreciable serum levels. Bile acids were not found in the urine of most patients with Laennec's cirrhosis. The presence of bile acid in the urine of two cirrhotic subjects (Cases Nos. 18 and 19) may be related to a moderate proteinuria which was present in both of these patients.

The mechanism of renal clearance of these substances appears to be glomerular filtration followed by extensive tubular reabsorption. At a serum concentration of 5 mg. per cent approximately 76 per cent of TBA and 95 per cent of DBA is bound to serum albumin. Only the unbound serum bile acid would be filtered by the glomeruli. On the basis of serum concentrations, binding constants (15) and urinary bile acid content, it was calculated that more than 95 per cent of the filtered bile acid was reabsorbed by the tubules in several patients with obstructive jaundice.

Preliminary observations have indicated that the bile acids in both the urine and serum of patients with obstructive jaundice are largely in conjugated form. In patients with Laennec's cirrhosis, only a small portion of the serum bile acid is conjugated. Differences in the state of conjugation of the bile acids may affect their renal tubular reabsorption. Further study of the nature of the bile acid conjugates in serum and urine will be necessary, in order to understand the differences in urinary excretion of bile acids in these patients.

SUMMARY

In normal subjects, bile acids were not detectable in the serum or urine. In 12 of 13 patients with Laennec's cirrhosis, 0.5 to 5.5 mg. per cent of dihydroxy bile acid (DBA) was present in the serum. In only two of these patients was trihydroxy bile acid (TBA) detectable (0.4 to 0.6 mg. per cent). Only a small proportion of the serum dihydroxy bile acid from patients with Laennec's cirrhosis was conjugated. Impaired activity of the conjugating and hydroxylating enzymes within

the liver cells probably accounts for the serum bile acid findings in this group.

All of 10 patients with biliary obstruction were found to have an accumulation of both tri- and dihydroxy bile acids in the serum. The ratio of tri- to dihydroxy acid varied from 1 to 4. In these patients the serum bile acids were largely conjugated with glycine and taurine, and probably accumulated in the blood as a result of regurgitation from the biliary passages.

The following correlations were observed between serum bile acids and the other findings of hepatic disease in these patients:

1) The serum TBA/DBA ratio indicated the relative intensities of biliary regurgitation and of hepato-cellular injury in the hepatic disorder.

2) Serum DBA concentration exhibited a correlation with serum bilirubin in both hepatocellular and regurgitative jaundice.

3) In patients with a component of biliary obstruction, the serum TBA concentration was proportional to the hypercholesterolemia.

Bile acids were regularly excreted in the urine by patients with obstructive jaundice, but were not detected in the majority of patients with Laennec's cirrhosis. Differences in the state of conjugation of the bile acids appear to affect the renal clearance of these substances.

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BILE ACID CONTENT OF HUMAN SERUM. II. THE BINDING OF CHOLANIC ACIDS BY HUMAN PLASMA PROTEINS [1]

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Although many observations indicate that bile acids and their derivatives may be bound by serum proteins, little information upon the quantitative aspects of this reaction is available. Lecomte du Noüy (1) observed that the activity of bile salts in lowering the surface tension of aqueous solutions was suppressed by the presence of serum.

The activity of human albumin in preventing the lysis of red blood cells by the bile salts was believed by E. J. Cohn to indicate the binding of these substances by albumin (2). The bile acids which are contained in the serum of patients with hepatic disease have been found in this laboratory to be largely non-dialyzable (3). These observations indicate that these substances are bound by serum proteins. This binding to proteins is a factor which may influence the serum concentration and renal clearance of the bile acids, as well as their relationship to such other serum constituents as the lipids and bilirubin. The present report is concerned with the binding of several bile acids by certain plasma protein fractions, as measured by the dialysis-equilibrium method, and with the effect of pH upon this reaction.

TABLE I

Binding of bile acids by human plasma protein fractions

Plasma protein fraction* [Cohn (4)]	mM × 10^{-3} of bile acid bound by 100 mg. of protein			
	Deoxycholic acid	Cholic acid	Glycodeoxycholic acid	Glycocholic acid
I	0	0		
II	0	0		
III	1.5	0	1.6	0
IV-1	1.8	0	1.3	0
V	3.3	1.4	3.3	1.3

* Major components of these fractions are as follows: I, fibrinogen; II, γ-globulin; III, β-globulins; IV-1, α-globulins; V, albumin.

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[2] Fellow of the New York Heart Association.

MATERIALS AND METHODS

Studies were made of the amount of bile acid bound by the different fractions of human serum protein isolated by Cohn's method (4). Ten ml. of a 1 per cent solution of each fraction in buffer (0.15 M NaCl, 0.01 M Na phosphate, pH 7.6) was placed in a small cellophane dialysis sac and equilibrated at 5° C for 48 hours against 50 ml. of a solution of the sodium salt of a cholanic acid dissolved in the same buffer. At the same time 10 ml. of buffer were equilibrated against 50 ml. of the solution of cholanic acid.[3]

After equilibration with buffer or serum protein, the bile acid concentration in the outer solution was determined spectrophotometrically (5, 6) on an aliquot of from 1 to 3 ml.[4] The concentration of cholanic acid in the outer solution, which represents also the concentration of unbound bile acid within the sac, makes it possible to calculate the total quantity of unbound bile acid. By subtraction of unbound bile acid from the total bile

[3] The dialysis tubing was boiled three times and rinsed repeatedly with distilled water prior to use.

The equilibration was virtually complete after 24 hours. Increasing the time beyond 48 hours produced no further change in the concentration of the cholanic acids in the outside phase. When only buffer was placed in the sac, the concentration of cholanic acid after equilibration was identical in the inner and outer solutions, and was equal to the value calculated from dilution. There was no evidence of adsorption by the membrane itself.

[4] In the spectrophotometric determination of bile acid possessing one hydroxyl group, the 65 per cent sulfuric acid reagent must be replaced by a 9 to 1 mixture of concentrated H_2SO_4 and glacial acetic acid, as was first shown in the case of lithocholic acid by Minibeck (7). Monohydroxy bile acids possessing a double bond in the ring system, however, exhibit a suitable ultraviolet absorption spectrum in 65 per cent H_2SO_4 after heating at 60° for 15 minutes.

The contents of the sac were not analyzed since the presence of the protein decreases the exactness of the cholanic acid determinations.

acid content of the system, the quantity of bile acid bound by the protein within the sac was calculated.

The Donnan effect was considered to be of negligible magnitude and was disregarded in the calculations.

Commercial samples of deoxycholic acid, hyodeoxycholic acid and cholic acid were employed in these studies. The following compounds were kindly supplied by Dr. Erwin H. Mosbach: Taurine and glycine conjugates of deoxycholic and cholic acids; chenodeoxycholic acid; lithocholic acid; 7-hydroxy cholanic acid; 3-hydroxy 12-keto cholanic acid; 3, 7-dihydroxy 12-keto cholanic acid; and the formyl derivatives of deoxycholic and cholic acids. 3, 12-dihydroxy 7-keto cholanic acid was donated by Dr. Norman A. Hulme of Sterling-Winthrop Research Institute. 3-hydroxy, 12-keto, Δ9-11 cholenic acid was given by Dr. Karl Pfister of Merck Research Laboratories. The protein fractions of human plasma were generously donated by Dr. J. M. Ashworth of the American Red Cross through E. R. Squibb and Sons.

RESULTS

Binding of bile acids by various plasma proteins

The extent of binding of deoxycholic and cholic acids, by 100 mg. of each of 5 plasma protein fractions, is indicated by Table I. The protein, in a volume of 10 ml., was equilibrated against 12.7×10^{-3} mM of bile acid in a volume of 50 ml. Among the plasma proteins albumin exhibits the greatest binding activity towards both of these bile acids. The uptake of deoxycholic and glycodeoxycholic acids by Fractions III and IV-1 was approximately half that shown by albumin. Fibrinogen and gamma globulin did not bind either deoxycholic or cholic acid.

Extent of binding by albumin of the series of cholanic acids

The chemical structure of the bile acid was found to influence the extent to which it is bound to human serum albumin.

Ten ml. of a 1 per cent human serum albumin solution (1.4×10^{-3} mM of albumin) was equilibrated with 50 ml. of buffer containing 12.7×10^{-3} mM of a number of bile acids and their derivatives. Table II lists the bile acids and derivatives studied, together with the number of moles of the bile acid bound by each mole of albumin under these conditions. The extent of binding decreases as the number of hydroxyl groups on the ring system is increased. It was greatest for the bile acids with a single hydroxyl group and least for cholic acid, which has three hydroxyl groups. The position of the hydroxyl groups in the ring system has little influence upon the degree of binding. Neither conjugation of the carboxyl group with glycine or taurine, nor covering up the hydroxyl groups by the formyl radicals greatly changed the extent of binding.

The introduction of a keto group in either the 7 or 12 position of the ring system suppresses the affinity for albumin. In the only unsaturated compound tested (3-hydroxy, 12-keto, Δ9-11 cho-

TABLE II

Binding of various cholanic acids by human serum albumin

Common name	Structure	Moles of cholanic acid bound by one mole of albumin
Monohydroxycholanic acids		
Lithocholic acid	3-OH cholanic acid	6.5
	7-OH cholanic acid	6.7
Dihydroxycholanic acids		
Deoxycholic acid	3, 12 di-OH cholanic acid	2.3
Hyodeoxycholic acid	3, 6 di-OH cholanic acid	3.2
Chenodeoxycholic acid	3, 7 di-OH cholanic acid	3.0
Trihydroxycholanic acids		
Cholic acid	3, 7, 12 trihydroxy-cholanic acid	.94
Conjugated cholanic acids		
Glycodeoxycholic acid	3, 12 di-OH cholanyl glycine	2.3
Taurodeoxycholic acid	3, 12 di-OH cholanyl taurine	2.3
Glycocholic acid	3, 7, 12 tri-OH cholanyl glycine	.92
Taurocholic acid	3, 7, 12 tri-OH cholanyl taurine	.59
Ketocholanic acids		
—	3-OH, 12-keto cholanic acid	0*
—	3, 7 di-OH, 12-keto cholanic acid	0
—	3, 12 dihydroxy 7-keto cholanic acid	0*
— (Unsaturated)	3-OH, 12-keto, Δ 9–11 cholenic acid	1.6
Formylated cholanic acids		
Diformyldeoxycholic acid	Diformyl 3, 12 di-hydroxy cholanic acid	3.2
Triformylcholic acid	Triformyl 3, 7, 12 tri-hydroxy cholanic acid	1.2

* With larger amounts of albumin, it is possible to demonstrate slight binding of these two cholanic acid derivatives by albumin.

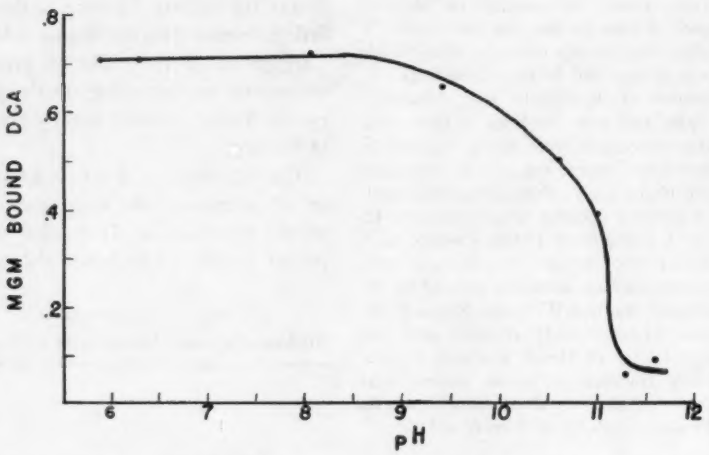

FIG. 1. THE BINDING OF DEOXYCHOLIC ACID BY 50 MG. OF HUMAN ALBUMIN AT VARIOUS pH'S

Five ml. of 1 per cent albumin solution in buffer was dialyzed against 2.5 mg. of sodium deoxycholate in a volume of 25 ml. of buffer.

lenic acid), the introduction of a double bond into the ring system increased the extent of binding with albumin.

Effect of pH on binding reaction

The effect of hydrogen ion concentration upon the interaction with albumin was studied, in the case of deoxycholic acid, over a pH range of 5.9 to 11.6 (Figure 1). The reaction is indifferent to hydrogen ion concentration until the pH of 9.0 is reached. Above pH 9.0 the affinity for albumin decreases rapidly, and is nearly absent above pH 11.0. Denaturation of albumin by alkaline pH was excluded by the finding that the albumin solutions, after the experiment had been completed, exhibited the usual uptake of deoxycholate at pH 7.6.

Calculation of binding constants

It is apparent from Table II that each molecule of albumin can bind more than one molecule of cholanic acid. The number of molecules of cholanic acid bound by one molecule of albumin is determined by the concentration of unbound cholanic acid. The relationship between bound and unbound cholanic acid involves two parameters: the dissociation constant of the albumin-bile acid complex (K) and the maximum binding capacity of the albumin molecule (n).

If it is assumed that in a molecule the size of albumin, the reactivity of a binding site is not markedly affected by the state of other binding sites, then the reaction can be treated as a simple bi-molecular reaction between the binding site and the cholanic acid.

The equilibrium conditions of this reaction may be formulated as follows (8):

$$1/r = K/n\ 1/(A) + 1/n$$

where r is the ratio of moles of bound cholanic acid to moles of albumin; A is molar concentration of unbound cholanic acid; n is maximum moles of cholanic acid which can be bound by one mole of albumin; and K is dissociation constant of the albumin-cholanic acid complex. It is apparent that a plot of 1/r as a function of 1/A should assume a linear form, the vertical intercept representing 1/n and the slope representing K/n. By this method, the constants K and n may be calculated for each bile acid.

Such data have been obtained for deoxycholic acid and cholic acid (Figure 2). Solutions of the sodium salt of each bile acid in buffer, were prepared with a bile acid concentration of 1.27×10^{-4}, 2.54×10^{-4}, 5.08×10^{-4}, and 12.70×10^{-4} mM

per ml. Fifty ml. of each solution were equilibrated with 10 ml. of a 1 per cent solution of albumin in buffer. Measurement of unbound bile acid concentration in the outer solution after equilibration, and calculation of quantity of bile acid bound by the albumin within the sac, provided the data for the curves in Figure 1. From the intercept and slope of these curves, it may be calculated that for deoxycholic acid, $K = 7.4 \times 10^{-4}$ and $n = 12$, for cholic acid, $K = 6.5 \times 10^{-4}$ and $n = 4$.

DISCUSSION

The binding of the cholanic acid series of compounds by serum albumin might have been predicted from knowledge of the interaction of serum albumin with long chain fatty acids and with a variety of other organic anions (9). Serum albumin has been considered to be unique among the plasma protein fractions in its ability to bind organic anions (10). The present data confirm the preeminence of albumin in this regard, but indicate that the lipoprotein-containing Fractions III and IV-1 also interact with the cholanic acids, although to a lesser extent than does albumin.

The pH effect, namely the suppression of binding above pH 9, is compatible with the existence of an electrostatic bond between the positively charged ε-amino group of lysine (pK 9.3), and the negatively charged carboxylate group of the cholanic acid, as the primary force responsible for the binding. This finding parallels that of Klotz and Walker in the binding of methyl orange by bovine serum albumin (11).

However, the existence of secondary forces between albumin and the bile acid is indicated by the variation in extent of binding among closely related cholanic acids (Table II). The data indicate that the polarization of the cholanic acid ring system by the successive introduction of hydroxyl groups suppresses the affinity for albumin.

There are three times as many sites in albumin available for binding deoxycholic acid, a dihydroxy compound, as are available for binding cholic acid, which possesses 3 hydroxy groups. These observations are consistent with the postulated role of van der Waal forces acting upon the non-polar region of the smaller molecule, in determining the affinity of the substance for albumin

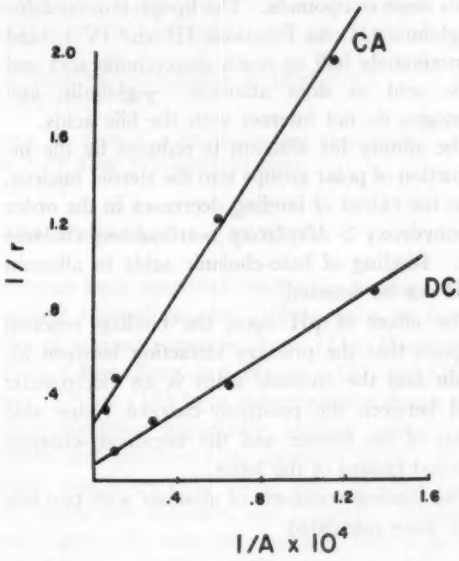

FIG. 2. BINDING OF DEOXYCHOLIC ACID (DCA) AND CHOLIC ACID (CA) BY HUMAN ALBUMIN AS A FUNCTION OF CONCENTRATION OF THE UNBOUND ACID

(10). The marked reduction of affinity for albumin which results from the presence of a keto group in the ring system, likewise appears to be caused by changes in the polarity of the ring system.

The cyclopentano-perhydrophenanthrene ring system of the bile acids is also found in such biologically important substances as cholesterol, steroid hormones, and cardiac glycosides, which differ from one another in the structure of the ring system and in the nature of substituent groups upon the ring. Study of how the interaction of cholanic acids with albumin is modified by changes in the structure of the cholanic acids may yield information of interest. The testing of additional cholanic acid derivatives will provide further information on the relationship between molecular structure and affinity for the various plasma proteins.

SUMMARY

The binding of bile acids and their derivatives by the protein fractions of human plasma has been studied by the dialysis-equilibrium method. Albumin exhibits the greatest binding activity to-

wards these compounds. The lipoprotein-containing globulins, Cohn Fractions III and IV-1, bind approximately half as much deoxycholic acid and cholic acid as does albumin. γ-globulin and fibrinogen do not interact with the bile acids.

The affinity for albumin is reduced by the introduction of polar groups into the steroid nucleus. Thus the extent of binding decreases in the order monohydroxy > dihydroxy > trihydroxy cholanic acid. Binding of keto-cholanic acids to albumin could not be detected.

The effect of pH upon the binding reaction suggests that the primary attraction between albumin and the cholanic acids is an electrostatic bond between the positively-charged lysine side chains of the former and the negatively-charged carboxyl groups of the latter.

The binding constants of albumin with two bile acids were calculated.

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INHIBITORY EFFECT OF CHLORPROMAZINE UPON THE ADRENAL CORTICAL RESPONSE TO INSULIN HYPOGLYCEMIA IN MAN [1]

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(Submitted for publication October 12, 1956; accepted December 13, 1956)

The large number of recent publications concerning the inter-relationships between the central nervous system and the anterior pituitary testifies to the intense interest in this subject (2, 3). Particular attention has been devoted to the effects of hypothalamic stimulation or injury upon pituitary ACTH release and adrenal cortical response. The work of deGroot and Harris (4), Hume and Wittenstein (5), Porter (6), McCann (7), and many others has indicated the importance of hypothalamic integrity in the normal release of adrenocorticotropin from the pituitary in response to a stimulus.

Another approach to the problem of neural control of the pituitary has been made through the use of central nervous system-depressant drugs such as morphine (8), which reportedly exerts an inhibitory influence upon pituitary release of corticotropin in response to acute stressing procedures. Among the drugs so used is the phenothiazine derivative, 10-(γ-dimethylaminopropyl)-2-chlorophenothiazine ("largactil," chlorpromazine). A major site of action of this agent has been thought to be the hypothalamus (9). Some weight was lent to this supposition by the finding of Wase, Christensen, and Polley that chlorpromazine labeled with S^{35} was accumulated in the hypothalamus in concentrations exceeding those attained in other areas of the brain (10).

Reports of the effect of this drug upon the adrenal response (and by inference, upon the hypothalamic-pituitary-adrenal response) to various stresses have described conflicting findings. Using rat adrenal ascorbic acid-depletion as the index of adrenal response, Aron (11), Hamburger (12), Ohler, Sevy, and Weiner (13), and Olling and deWied (14) have independently shown that chlorpromazine apparently blocked adrenal ascorbic acid depletion after operative shock. Holzbauer and Vogt (15) found that the drug failed to inhibit this response, and Cheymol, deLeeuw, and Oger (16) were able to show only a partial interference. Additional difficulties arose from the work of Georges and Cahn (17) who observed that chlorpromazine could itself produce eosinopenia in rats; of Egdahl, Richards, and Hume (18) who demonstrated elevations of cortisol levels in adrenal venous blood of intact dogs after intravenous chlorpromazine administration; of Hamburger (12) who showed moderate ascorbic acid depletion after chlorpromazine alone; and of Harwood (19) who reported rises in plasma 17-hydroxycorticosteroid values in monkeys given the drug.

In the face of these conflicting data, the following study was undertaken in an attempt to clarify the effects of chlorpromazine on the adrenocortical response of human subjects to acute stimuli.

METHODS AND MATERIALS

1. *Experimental procedure.* The stimulus employed in this study was insulin coma as used in the treatment of patients with schizophrenia. The index of pituitary-adrenal response was the rise in plasma 17-OH-corticosteroid levels. Such rises have been reported to occur even after mild insulin hypoglycemia (20). Plasma steroid responses to insulin in schizophrenic patients were shown to be entirely comparable to the normal by Bliss, Migeon, Branch, and Samuels (21).

The procedure was as follows. Nine patients, seven females and two males, aged 15 to 44, who were undergoing insulin coma treatment for schizophrenia were selected. Control blood samples for plasma corticosteroid

[1] Supported in part by a grant to Dr. Joseph W. Jailer from the National Institute of Arthritis and Metabolic Diseases (A-195[C]). These data were presented in preliminary form at the 38th Annual Meeting of the Endocrine Society, June, 1956 (1).

[2] John and Mary R. Markle Scholar in Medical Science.

[3] Dickenson Traveling Fellow, University of Manchester, England.

determinations were drawn at 6:30 a.m. Standard insulin was then administered subcutaneously in doses of 45 to 670 units. Two additional blood samples were then drawn, one at the beginning of coma, and the second just before its termination (*i.e.*, at 3 and 4 hours after insulin).

Four to 21 days later the same procedure was repeated after pre-treatment at 4:30 a.m. and at 6:30 a.m. with 50 to 150 mg. of chlorpromazine hydrochloride given orally, for total doses of 100 to 300 mg. This timing was selected in an effort to attain maximal blood levels of the drug at the inception and at the height of coma (9).

Three to 17 days after the chlorpromazine experiment was performed in a given patient, the procedure was repeated without chlorpromazine.

In eight of the nine patients, blood specimens for glucose determination were obtained at 0 time (6:30 a.m.) and 3 hours after insulin administration.

2. *Effect of chlorpromazine upon response of the adrenal cortex to exogenous ACTH.* In order to rule out the possibility that chlorpromazine might exert an effect upon adrenal cortical response to ACTH, patients treated with the drug in large doses (400 to 1,000 mg. per day) for periods of 25 to 70 days were subjected to standardized intravenous ACTH tests in a manner previously described (22), the index of response again being the rise in plasma 17-OH-corticosteroid levels.

In addition, three patients received chlorpromazine acutely, with a dose schedule like that employed in the insulin coma studies, and then were subjected to intravenous ACTH tests.

3. *Analytical methods.* Levels of plasma 17,21-dihydroxy-20-ketosteroids were estimated by the Silber-Porter method (23), as modified in this laboratory (24), and more recently, by a procedure which incorporated the essential features of the changes made by Peterson, Wyngaarden, Guerra, Brodie, and Bunim (25) and which eliminates the need for adding cortisol to the unknowns.

Blood glucose determinations were made according to the method of Benedict (26).

For more specific measurement of plasma 17-OH-corticosteroids, paper chromatography was resorted to. This was deemed necessary because it was noticed as the study progressed that a pink color formed in the blank tubes of plasma samples taken from patients who had received large doses of chlorpromazine. *In vitro* studies revealed that this chromogen was due to chlorpromazine and that it also interfered with optimal development of Porter-Silber chromogen, *i.e.*, with the development of the color reaction between cortisol and phenylhydrazine-sulfuric acid. Readings were reduced, at most, to values 15 per cent below control levels in *in vitro* experiments in which chlorpromazine in amounts equivalent to 250 micrograms per 100 ml. plasma was added to known amounts of cortisol.[4] Attempts to separate chlorpromazine from cortisol *in vitro* and *in vivo* by washing extracts with 1 N sulfuric acid (9) and by means of column chromatography (18) were not successful.

Separation was achieved by paper chromatography. Plasma was extracted with ethyl acetate, washed with base, subjected to hexane: methanol partition, and the dried extract chromatographed in a formamide: chloroform system as described by Burton, Zaffaroni, and Keutmann (27). The cortisol region was detected by ultraviolet light absorption and mobility in comparison with a simultaneously chromatographed reference standard of steroid. Quantitation of cortisol (and further confirmation of its probable identity) was carried out after methanol elution from paper by the colorimetric methods of Porter and Silber (28), and (in one instance) Gornall and Macdonald (29). In each case, blanks (sulfuric acid and ethanol without phenylhydrazine or 2,4-dinitrophenylhydrazine) were run on the eluate of the cortisol region of the paper chromatogram. The fact that no pink color was detectable in any of these blanks was taken as evidence that the chlorpromazine had been separated from the steroid. In the 11 samples tested by the Porter-Silber method (28), unknowns formed the usual yellow color with an absorption maximum at 410 millimicra. Corresponding blanks did not give inordinately high readings (4 to 25 per cent of the readings of unknowns) either in the chlorpromazine-treated specimens or in those which were untreated.

Recoveries of known amounts of cortisol ranged from 35 to 75 per cent by this technic. *In vitro* addition of chlorpromazine in amounts as large as 100 micrograms did not alter recoveries of cortisol.

Plasma samples which were subjected to these extraction and chromatographic procedures were obtained from 12 additional patients with insulin coma. Large samples of blood were drawn at 3 and at 4 hours, and the two specimens from a given patient pooled together. Quantitation of post-insulin cortisol levels in the group receiving only insulin was compared with quantitation of the steroid in the group receiving insulin plus chlorpromazine.

[4] No reliable quantitative data are yet available concerning the plasma levels of chlorpromazine attained in human subjects given the usual doses of the drug (9). The figure, 250 micrograms per 100 ml. plasma, was arrived at after trial and error as a quantity which produced the pink chromogen to a degree greater than that encountered in plasma specimens obtained from the chlorpromazine-treated subjects. Plasma concentrations in the patients were therefore assumed to be less than 250 micrograms per 100 ml. The validity of that assumption appears to be borne out by data presented in a study published since the preparation of this report (Salzman, N. P., and Brodie, B. B., Physiological disposition and fate of chlorpromazine and a method for its estimation in biological material. J. Pharmacol. & Exper. Therap., 1956, **118**, 46). Dogs receiving 20 mg. per kg. of chlorpromazine intravenously attained plasma levels of 70 to 260 micrograms per 100 ml. during the period, 0.5 to 3 hours after administration. The doses given to the subjects in the present study were of the order of 1.5 to 4.5 mg. per kg.

TABLE I

Response of plasma 17-OH-corticosteroid levels to insulin hypoglycemia
Initial control experiments

Patient	Insulin (*units*)	Plasma 17-OH-corticosteroids (*micrograms %*)		
		Control	3 Hours	4 Hours
J. S.	420	23	37	43
M. K.	390	21	26	39
J. Sc.	500	27	41	53
S. L.	160	19	33	34
R. M.	270	19	48	46
S. R.	50	26	33	45
J. D.	630	20	29	30
I. S.	100	14	27	40
H. M.	320	22	33	44
Average	316	22	34	42

TABLE II

Effect of chlorpromazine upon plasma 17-OH-corticosteroid response to insulin hypoglycemia

Patient	Insulin (*mg.*)	Chlorpromazine (Total dose)	Plasma 17-OH-corticosteroids (*micrograms per 100 ml.*)		
			Control	3 Hours	4 Hours
J. S.	400	300	30	23	27
M. K.	410	200	27	10	23
J. Sc.	170	200	23	14	7
S. L.	160	100	27	14	13
R. M.	300	300	22	0	27
S. R.	45	200	15	8	8
J. D.	630	200	11	6	19
I. S.	120	200*	23	15	22
H. M.	300	300	4	8	11
Average	281		20	11	17

* A dose of 150 mg. chlorpromazine failed to suppress adrenocortical response to insulin hypoglycemia in this patient.

RESULTS

Response of plasma 17-OH-corticosteroid levels to insulin coma

Effects of insulin coma upon plasma 17-OH-corticosteroid values are summarized in Table I. It will be seen that resting levels fell within the normal range (4 to 28 micrograms per 100 ml. [24]), and at 3 and at 4 hours after insulin there was a consistent rise to mean levels of 34 and 42 micrograms per 100 ml., respectively. These levels were above the normal range and were roughly comparable to levels attained by normal individuals following intravenous administration of 25 i.u. ACTH (22). There appeared to be no correlation between the magnitude of plasma 17-OH-corticosteroid rise and the size of the dose of insulin.[5]

Effect of chlorpromazine upon plasma 17-OH-corticosteroid response to insulin coma

Table II shows the results in the same patients pre-treated with chlorpromazine. It is apparent that the resting levels of plasma 17-OH-corticosteroids were comparable to those found in the control experiments (mean levels, 20 and 22 micrograms per 100 ml., respectively). In contrast to the control responses, 17-OH-corticosteroid levels during coma did not rise, but remained within normal limits. The trend of values was reminiscent of the normal diurnal variation of plasma 17-OH-corticosteroid levels observed by Bliss, Sandberg, Nelson, and Eik-Nes (30). The duration and depth of coma did not appear to differ from the coma seen during the control experiments without chlorpromazine. The only clinical differences noted were diminution of salivation and sweating in the chlorpromazine-treated group. No differences in depression of blood pressure were found.

In the repeat control experiments, plasma 17-OH-corticosteroid levels rose as they had in the initial controls in six of eight subjects (Table III). The data from control and chlorpromazine experiments in four of the patients are presented graphically in Figure 1.

In two additional patients, chlorpromazine did not clearly inhibit the adrenal cortical response to insulin. Since the control rises in plasma 17-OH-corticosteroid levels were also equivocal, it was thought reasonable to exclude the findings in these cases from the data presented.

As shown in Table IV, depressions of blood glucose in the control and chlorpromazine studies did not differ appreciably.

Chromatographic studies of plasma 17-OH-corticosteroids during insulin coma

Table V summarizes the results of chromatographic fractionation and quantitation of plasma cortisol levels. In the Table are compared the

[5] This dose was arrived at by purely empirical means, being the dose required to produce satisfactory coma which was not unduly prolonged. The required dose was extremely variable from patient to patient, and tended in most cases to become progressively smaller during the course of the series of coma treatments.

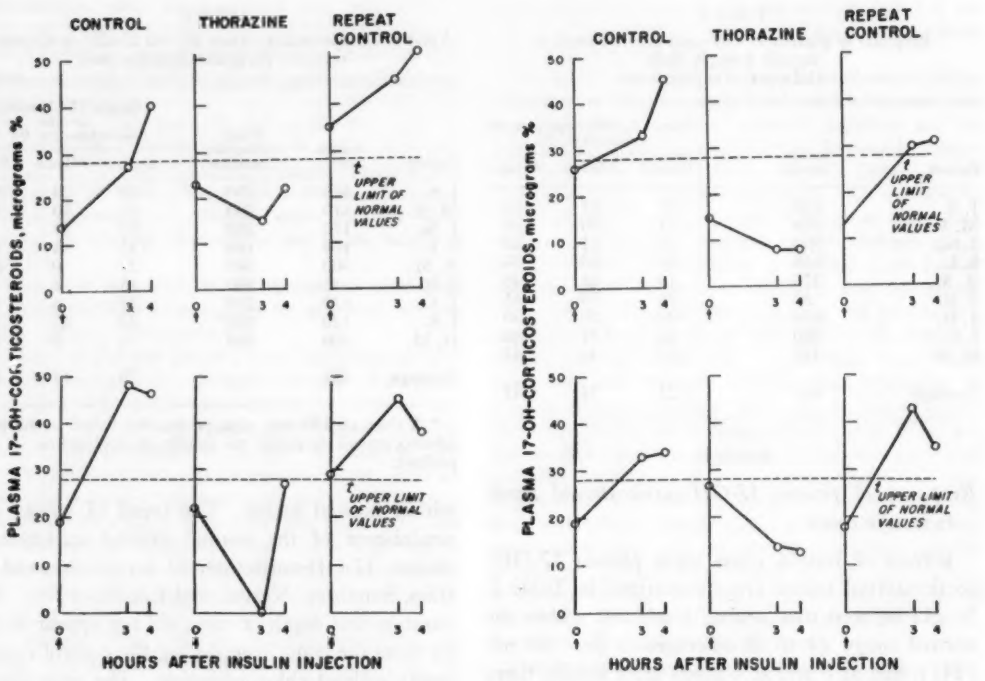

FIG. 1. EFFECT OF CHLORPROMAZINE UPON ADRENOCORTICAL RESPONSE TO INSULIN HYPOGLYCEMIA

Arrows indicate time of insulin injection. Data obtained from patients I. S., S. R., R. M., and S. L. (reading left to right).

amounts of cortisol detected during insulin coma with and without chlorpromazine pre-treatment. Without chlorpromazine, the mean value for plasma cortisol after chromatography was 18.0 micrograms per 100 ml. (without chromatography, 34.6 micrograms per 100 ml.; "recovery," 52 per cent). In the chlorpromazine-treated subjects, the average value was 8.7 micrograms per 100 ml. (without chromatography, 16.7 micrograms per 100 ml.; "recovery," 52 per cent). The difference between the means of the two groups was statistically significant ($P < 0.01$). The fact that about the same proportion of cortisol was recovered in control and chlorpromazine experiments after chromatography lessened the suspicion that the difference in cortisol levels could be accounted for by an adverse effect of chlorpromazine itself upon steroid recovery. This already seemed unlikely from *in vitro* studies (cf. Section 3, *Methods and Materials*).

Effect of chlorpromazine upon plasma 17-OH-corticosteroid response to exogenous ACTH

Table VI shows the effects of long- and short-term chlorpromazine administration upon the response of the adrenal cortex to exogenous ACTH. It is obvious that only one of the patients (A. T.) showed a very slightly reduced response. Despite the inhibitory effect of the drug upon plasma corticosteroid response to insulin, it did not bring about a suppression of ACTH-responsiveness of the adrenal cortex as does prolonged steroid administration (31). Similarly, acute administration of the drug, given according to the dose schedule used in the insulin studies, had no suppressive effect upon the response to ACTH.

DISCUSSION

The data presented confirm the efficacy of insulin hypoglycemia as a stimulus to the adrenal cortex in man (20). The results also indicate

TABLE III

Response of plasma 17-OH-corticosteroid levels to insulin hypoglycemia

Repeat control experiments

Patient	Insulin (*units*)	Plasma 17-OH-corticosteroids (*micrograms %*) Control	3 Hours	4 Hours
J. S.				
M. K.	400	37	48	48
J. Sc.*	190	26	27	38
S. L.	160	18	43	35
R. M.	200	29	45	38
S. R.	45	14	30	31
J. D.	670	29	31	34
I. S.	120	35	45	51
H. M.	110	22	17	25
Average	237†	26	36	38

* This patient received 100 mg. phenobarbital prior to experiment.

† Note that average dosage of insulin is less than in the chlorpromazine experiment (Table II), yet adrenocortical response still occurs in a manner comparable to initial controls (Table I).

that chlorpromazine can inhibit the adrenocortical reaction to this stimulus. The inhibition of plasma 17-OH-corticosteroid rise after insulin which was caused by chlorpromazine does not seem to be an artifact. Chromatographic separation and quantitation of steroid revealed that cortisol in plasma of the chlorpromazine-treated subject was reduced by about 50 per cent in comparison with the control. This was essentially the same degree of reduction found in unchromatographed plasma specimens.

TABLE IV

Lack of influence of chlorpromazine upon blood glucose response to insulin hypoglycemia

Patient*	Blood glucose (*mg. per 100 ml.*) Without chlorpromazine Control	3 Hours	With chlorpromazine Control	3 Hours
M. K.			70	30
J. Sc.	97	42	101	39
S. L.			87	29
R. M.	97	30	103	31
S. R.	82	33	94	42
J. D.			103	31
I. S.			69	19
H. M.	94	32	97	34
Average	93	34	91	32

* In all but one of the patients in whom blood glucose responses were recorded both with and without chlorpromazine, insulin doses were comparable.

TABLE V

Results of chromatographic analysis of cortisol present in plasma of patients during insulin coma, with and without chlorpromazine pre-treatment

Specimen	17-OH-corticosteroids (*micrograms per 100 ml.*) Without chlorpromazine Before chromatography	Without chlorpromazine After chromatography*	With chlorpromazine Before chromatography	With chlorpromazine After chromatography*
1	32.5	21.0		
2		16.4		
3	38.2	12.9		
4	34.9	11.4		
5	36.0	26.0		
6	31.2	20.0		
7			15.2	7.6
8			15.3	9.8
9			20.9	11.2
10			12.3	10.0
11			21.6	8.0
12			15.0	5.8
Average	34.6	18.0†	16.7	8.7†
S.D.		5.5†		1.9†
Cortisol recovered after chromatography (Average, %)		52		52

* Values after chromatography have not been corrected for losses.

† "T-test" shows statistically significant difference between the mean values ($P < 0.01$).

The results appear to support those of certain previous investigations in animals (11–14). The absence of definite blockade of adrenal cortical response reported by other workers (15–17) may perhaps be explained by differences in dosage and

TABLE VI

Lack of inhibitory effect of chlorpromazine upon response of plasma 17-OH-corticosteroid levels to a standard intravenous ACTH test

Patient	Chlorpromazine dose/day (*mg.*)	Duration of treatment (*days*)	Plasma 17-OH-corticosteroids (*micrograms per 100 ml.*) Before ACTH	After ACTH
A. E.	400	25	25	59
A. T.	600–1000	30	17	33
H. G.	1000	30	41	63
R. D.	400–1000	70	19	47
J. D.	200	*	12	37
S. R.	200	*	11	37
M. G.	200	*	11	66
Normal range (31)			4–23	35–59

* Chlorpromazine administered in these three patients according to the dose schedule employed in the insulin coma experiments.

in timing of administration of the drug in relation to the stimulus imposed, as Hamburger has suggested (12). The findings presented here and earlier (11–14) are not necessarily incompatible with the adrenocortical-stimulating property of chlorpromazine demonstrated by Hamburger (12), by Egdahl, Richards, and Hume (18), and by Harwood (19). The factors of speed and route of administration may play a role, and as Harris has pointed out, chlorpromazine may be to some extent a toxic compound like other "blocking agents" which have been used in similar experiments and which themselves cause corticotropin release (2).

This experiment does not elucidate the mechanism of the demonstrated blocking action of chlorpromazine. It seems clear from the findings presented here and from the work of Olling and deWied (14) that the drug does not interfere with the action of adrenocorticotropin upon the adrenal cortex itself. There is no experimental precedent to suggest that this agent can cause an increased rate of disappearance of cortisol from plasma, or accelerated hepatic reduction and conjugation of cortisol. To date, an increased rate of cortisol disposal has been demonstrated only in hyperthyroidism (25).

In trying to visualize the mechanism of the blockade, one is tempted to assume that the site of action of chlorpromazine in inhibiting adrenocortical response (and by inference, pituitary corticotropin release) might be the hypothalamus. Some support for this assumption might be derived from the known suppressive effects of chlorpromazine upon the hypothalamus (9), from the moderately selective accumulation of chlorpromazine in hypothalamic structures (10), and from the knowledge that experimental damage to certain hypothalamic nuclei may result in inhibition of ACTH release in response to a stimulus (4–7). However, such a chain of reasoning must be viewed as entirely speculative, and it should be realized that the data presented in this report, which might be construed as supporting such a concept, constitute only indirect and circumstantial evidence.

The fact that relatively prolonged administration of chlorpromazine failed to suppress plasma 17-OH-corticosteroid response to exogenous ACTH (in contrast to long-term steroid administration [31]) is not necessarily incompatible with the inhibitory effect of the drug upon adrenal cortical response to an acute stimulus. The two apparently contradictory findings may perhaps be reconciled by Harris' concept of a dual control of the central nervous system over corticotropin release, one mechanism operating under quiescent conditions, the other under conditions of "stress" (2). Such an explanation is again speculative, and it is emphasized that the above discrepancy cannot be satisfactorily accounted for on the basis of present information.

Finally, it is perhaps of some importance that, however produced, blockade of adrenal cortical response to the rather severe stress of insulin hypoglycemia did not result in any clinical symptoms suggesting adrenal cortical insufficiency. Similar observations were made when adrenal cortical response to typhoid vaccine administration was prevented by blocking the pyrogenic reaction with aminopyrine (32). These findings may be interpreted as supporting the concept of the "permissive" role of the adrenal cortex in homeostasis as stated by Ingle (33), and again raise a question as to the advantage for the organism of large increases in adrenal cortical activity in response to acute stimuli.

SUMMARY

1. Insulin coma was again shown to cause adrenal cortical response in man, as measured by rises in plasma 17-OH-corticosteroid levels.

2. Chlorpromazine usually inhibited this adrenal cortical response to insulin hypoglycemia.

3. Chlorpromazine had no inhibitory effect upon the response of the human adrenal cortex to administered adrenocorticotropin.

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RENAL REABSORPTION OF PHOSPHATE IN NORMAL HUMAN SUBJECTS AND IN PATIENTS WITH PARATHYROID DISEASE

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A number of studies in the dog and in man (1–6) have demonstrated a maximal renal tubular reabsorptive capacity for phosphate (TmP [3]). However, some investigators have denied that the reabsorption of phosphate conforms to Tm characteristics (7–9). These workers have suggested that phosphate reabsorption is conditioned by the rate of phosphate filtration such that a fairly constant proportion of the filtered phosphate is reabsorbed. In view of the conflicting evidence and the relative paucity of phosphate titration studies on man, experiments were performed in order to obtain information on the mechanism of phosphate reabsorption, the constancy of reabsorption in any one individual, and its variability in a group of subjects. The data were required for subsequent investigations designed to evaluate the influence of several factors on renal phosphate transport. The studies were performed on normal individuals and in patients with parathyroid disease.

It was found that in any given experiment a TmP was demonstrable. However, the variability in TmP seen in most of our normal subjects between consecutive periods of a single experiment and between experiments generally exceeded the variability reported for glucose Tm and for PAH Tm. Furthermore, the range of values for TmP within a group of normal individuals is so large that an average value has little meaning. Fluctuations in TmP in our subjects could not be consistently correlated with the level of calcium or phosphate in the diet.

METHODS

Subjects. Studies were carried out on ten normal subjects, three patients with post-thyroidectomy hypoparathyroidism, and one individual with hyperparathyroidism. The normal individuals included five male and three female volunteers aged 20 to 27 years, one 17-year-old male (C. H.) convalescing from a hip injury, but ambulatory during most of the studies, and a 26-year-old female (W. H.) hospitalized with a diagnosis of hypoparathyroidism, but found to have normal parathyroid function. The hypoparathyroid patients, a 37-year-old male and two females, aged 42 and 52, had not been treated previously with any medication other than calcium salts. The manifestations of hypoparathyroidism were severe in the male and mild in the females. The hyperparathyroid subject, a 72-year-old male, was subsequently cured by the removal of a parathyroid adenoma.

All subjects were hospitalized on a metabolism ward, and during many of the studies they received constant diets of calculated calcium and phosphate content. There were no restrictions on the patients' activities.

Design of experiments. Studies were begun about 9 a.m. with the patient fasted for 12 to 16 hours. One-half hour prior to the start of each study the subject ingested a liter of water and thereafter drank 200 ml. of wa-

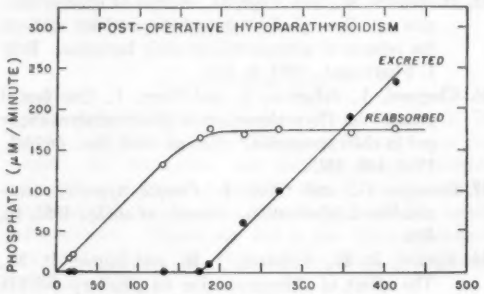

FIG. 1. THE QUANTITIES OF PHOSPHATE FILTERED, EXCRETED, AND REABSORBED DURING INTRAVENOUS PHOSPHATE LOADING IN A HYPOPARATHYROID SUBJECT

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[3] The following abbreviations will be used in this paper: P, inorganic phosphate; C_{In}, inulin clearance; GFR, glomerular filtration rate; RPF, effective renal plasma flow; Tm, maximal rate of renal tubular reabsorption; Ca, calcium; K, potassium; PAH, para-amino hippurate.

TABLE I

Effect of elevation of plasma inorganic phosphate on the quantity of phosphate filtered, excreted, and reabsorbed *

Total concurrent time *min.*	Urine flow *ml./min.*	C_{In} *ml./min.*	C_{PAH} *ml./min.*	Plasma P *μM/ml.*	Urine P *μM/ml.*	Phosphate			P reab. / P filt.
						Filtered *μM/min.*	Excreted *μM/min.*	Reabsorbed *μM/min.*	
35–39	Prime containing inulin and PAH given								
39	Infusion I containing inulin and PAH begun at 3.2 ml. per min.								
90–101	11.2	127	525	0.91	0.02	116	0.2	116	1.00
101–116	11.3	130	541	0.93	0.02	121	0.2	121	1.00
118	Infusion II begun; sodium phosphate at 0.55 mM per min. plus inulin and PAH								
148–180	4.28	133	526	1.95	17.5	260	75	185	0.71
180–214	2.92	134	504	2.82	48.0	378	135	243	0.64
214–241	7.39	142	560	3.25	29.2	461	216	245	0.53
241–267	5.70	138	509	3.49	44.5	481	254	227	0.47

* Patient C. H., normal male subject, age 17.

ter every 20 to 30 minutes to maintain a water diuresis. Female subjects were catheterized with a six-hole rubber catheter which remained in place throughout the experiment. In the early studies the male subjects were also catheterized. However, it was found that these individuals were able to void on command; hence, catheterization was not performed in later experiments. After a sample of venous blood and one of urine were obtained for determination of inulinoid blank, a priming dose of inulin and PAH calculated to provide plasma levels of 20 and 2 mg. per cent, respectively, was given over a period of 3 to 5 minutes. Constant infusion of these substances dissolved in physiological saline solution was maintained by a pump. Thirty to sixty minutes after the sustaining infusion was begun, timed urine collection periods were started. The period varied in duration from 20 to 40 minutes, and at the midpoint of each, blood was drawn from an antecubital vein into a heparinized syringe, immediately centrifuged, and the plasma separated. To close each period urine was collected by spontaneous voiding or through the catheter followed by irrigation with 20 to 60 ml. of distilled water and about 30 ml. of air.

After two to four periods the infusion was changed to one containing buffered sodium phosphate, pH 7.40, in addition to inulin and PAH. A sufficient volume of a 0.5 M phosphate solution was incorporated in the infusion to provide for the delivery of 0.4 to 0.8 mM P per minute. Such an infusion was continued for sixty minutes before urine collections were again begun. This period was sufficient to allow elevation of the plasma P to the desired levels and to assure a relatively slowly increasing plasma P concentration during the collection periods. Studies were carried out for two to four periods at the elevated plasma P levels. In some experiments there followed a third infusion designed to elevate further the plasma P.

Dietary intake. The low, intermediate and high calcium diets contained 130, 800, and 1500 mg., respectively, daily. A low P intake was achieved by the administration of a diet containing 600 mg. of P daily, and, in several studies by administering, in addition, 35 ml. of aluminum carbonate gel, one hour after each meal and at bedtime. The intermediate P diet contained 1,200 mg., and the high P diets 1800 to 3,000 mg. P. In several experiments variations in Ca or P intake without changes in other dietary constituents were studied by the administration of a constant low Ca-low P diet supplemented at meal time with calcium lactate or 10 per cent phosphoric acid. The patient was placed on a given dietary regimen at least three days, and often more than a week prior to the renal function studies.

Chemical methods. Inulin was determined in plasma and urine by the method of Schreiner (10), PAH by the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber (11), inorganic P by the method of Fiske and Subbarow (12). Plasma Ca was determined by the method of Clark and Collip (13), and urine Ca by the method of Shohl and Pedley (14). Plasma K was determined on a flame photometer utilizing an internal standard.

Calculations. The clearances of inulin and PAH were taken as measures of GFR and RPF, respectively. Filtered P was calculated as the product of the inulin clearance and the plasma P. Excreted phosphate was calculated as the product of urine P concentration and urine flow. The difference between filtered and excreted P was considered to be the reabsorbed P.

RESULTS

In thirteen studies on four normal and one hypoparathyroid subjects P reabsorption was determined at endogenous plasma P levels and following stepwise elevations of the plasma P. In all instances a maximal rate of phosphate reabsorption was reached, and further elevation of plasma P resulted in no further increase in the rate of reabsorption. This result is shown for one normal subject in Table I. Following two

TABLE II

Variation of TmP in nine normals and three hypoparathyroid subjects

Subject	Date	Diet Ca mg./day	Diet P mg./day	GFR ml./min.	TmP μM/min.	TmP/GFR μM/ml.	Standard deviation of TmP μM/min.	Coefficient of variation %
Normal males								
C. H.								
2.00 M²*	3–25–54	1,500	1,800	147	199	1.35		
	4–6–54	130	600(B)†	142	222	1.56		
	11–4–54	130	3,000	148	186	1.26		
	11–10–54	1,570	600(B)	138	238	1.72		
	12–22–54	130	600	146	219	1.50		
	3–14–54	1,500	600	149	256	1.72		
	Mean			145	220	1.52	25.4	11.5
W. R.								
1.76 M²	8–12–54	2,000	2,800	114	122	1.07		
	3–24–55	800	1,200	112	75	0.67		
	4–4–55	130	600(B)	103	81	0.79		
	Mean			110	93	0.84	25.6	27.6
D. F.								
1.94 M²	1–3–55	610	1,015	115	102	0.89		
	2–7–55	610	1,015	99	73	0.74		
	Mean			107	88	0.81	11.3	12.9
J. N.								
1.91 M²	6–10–54		900(B)	109	179	1.64		
	6–22–54	High	High	121	175	1.45		
	Mean			115	177	1.54	2.7	1.5
M. L.								
2.10 M²	12–14–54	High	High	151	159	1.05		
L. B.								
1.91 M²	11–17–54	High	High	138	125	0.90		
Normal females								
R. H.								
1.78 M²	1–17–55	130	600	115	137	1.19		
	2–3–55	1,500	600	112	105	0.94		
	3–3–55	130	1,600	118	120	1.02		
	3–28–55	130	3,000	145	101	0.70		
	4–14–55	2,200	3,000	138	111	1.24		
	Mean			126	115	0.91	14.3	12.4
E. E.								
1.82 M²	5–25–55	130	600(B)	142	185	1.30		
	6–15–55	1,500	1,800	131	198	1.51		
	Mean			136	191	1.40	9.2	4.8
E. P.								
1.77 M²	3–21–55	Medium	Medium	96	108	1.15		
Hypo-parathyroid								
A. W.								
65 kg.	8–25–54	4,400	1,200	96	158	1.65		
	9–14–54	2,200	600(B)	88	163	1.85		
	10–20–54	3,300	600	84	172	2.04		
	1–10–55	130	600	95	179	1.88		
	2–21–55	2,200	700	110	149	1.35		
	Mean			95	164	1.75	11.7	7.1
L. D.	3–9–55	Inconstant		76	102	1.34		
B. C.	2–9–55	2,000	600	73	98	1.34		

* Surface area.
† (B) = Basaljel.

periods at endogenous plasma P levels, buffered sodium phosphate was administered, and the plasma P and, hence, the filtered P rose. The initial increase in filtered P noted in periods three and four (148 to 180 minutes, and 180 to 214 minutes) resulted in a rise in both reabsorbed and

excreted P. However, with the further elevation of plasma and filtered P, noted in periods five and six, no increase in reabsorbed P resulted, indicating that a maximal rate of reabsorption had been reached. This relationship between filtered and reabsorbed P is shown in Figure 1, which presents a similar study in a hypoparathyroid subject (A. W.). The constancy of P reabsorption at elevated plasma P levels resulted in a reduction in the ratio of filtered P/reabsorbed P with further increases in the filtered load. This was a consistent finding and is shown for one experiment in the last column of Table I. In both of these studies endogenous urinary P excretion was reduced by the prior administration of a low P diet and aluminum carbonate gel. The Tm phenomenon was also noted in patients not given aluminum carbonate gel.

Repeated determinations of TmP in the same individual over a period of weeks or months indicated considerable variability in most subjects. The mean values of TmP on different days for five normal subjects (C. H., W. R., D. F., J. N., R. H.) and one hypoparathyroid individual (A. W.) are shown in Table II. The standard deviation of the mean and the coefficient of variation are included as indices of the variability. The TmP values listed in Table II were obtained at different dietary levels of Ca and P. The relationship of dietary Ca and P to TmP was investigated. In two normal individuals, C. H. and R. H., intensive studies of the effect of changes in dietary Ca and P on TmP were made, and less complete studies were carried out on four other normal and one hypoparathyroid (A.W.) subjects. Analysis of the results indicated that variations of TmP from one period to another on a given day were about as great as the variations on different dietary regimens in all but two subjects (C. H. and E. P.). Since C. H. was one of the two subjects in whom an extensive study of the effects of changes in dietary Ca and P was made, these results are considered worthy of mention. The highest TmP values were obtained when the patient was on a high Ca-low P intake, (11–10–54, 3–14–55) and the next highest when on a low Ca-low P diet (4–6–54). These results are shown in Table II.

It is of interest that the individuals who showed the greatest variation in TmP between experiments also showed the largest fluctuations within a given experiment. That this does not appear to be due to technically poorer experiments or to overall fluctuations in renal function in these individuals is suggested by the fact that the inulin and PAH clearances did not reveal large changes from period to period. This is illustrated in two experiments on W. R., a normal subject (Table III). It can be seen that although the clearances of inulin and PAH were fairly constant, there is considerable fluctuation in TmP. These results are in contrast to the small variations in TmP in a given experiment noted in some of the other normal subjects. The data suggest that physiological variations in TmP of considerable magnitude may occur over short time intervals.

TABLE III

Variations of TmP with relative constancy of C_{In} and C_{PAH} in two experiments on a normal subject *

Date	Total concurrent time *min.*	C_{In} *ml./min.*	C_{PAH} *ml./min.*	TmP *μM/min.*
3–24–55	105–141	110	670	61
	141–171	111	652	74
	171–188	114	636	89
4–4–55	128–158	102	522	104
	158–182	101	566	95
	182–214	104	556	44

* W. R., 22-year-old normal male.

No consistent change in TmP was noted to occur in the course of an experiment. Although most of the experiments lasted from two to five hours, in two experiments on two normal subjects phosphate was infused for fourteen and eighteen hours. No appreciable change in TmP was noted during, or at the end of this period of time.

Plasma calcium was usually reduced when phosphate was infused, and the degree of reduction appeared to be correlated with the magnitude of the increase in plasma P. In the normal individuals the plasma Ca never fell below 8 mg. per cent, and at no time was tetany produced. In two of the hypoparathyroid individuals mild tetany did result during P infusions.

Although the plasma K was often lowered immediately after a P infusion was begun, there was little change thereafter, and no correlation could be made between changes in plasma K and variations of TmP.

The variability of TmP among the normal indi-

viduals cannot be accounted for by differences in body size (Table II). Calculations of TmP per unit of surface area did not appreciably alter the wide range of values for TmP. The average TmP for the three hypoparathyroid subjects falls within the broad range of values found for the normals (Table II). Insufficient data were obtained to determine whether the variability of TmP on repeated determinations was significantly different than in normal individuals. A single study on one hyperparathyroid subject revealed a mean TmP of 26 μM per min., a figure distinctly lower than that seen in the normal subjects.

No consistent relationship between GFR and TmP was noted (Table II). The variability of the ratio TmP/GFR is greater than that found for glucose (TmG/GFR) by Smith and his co-workers (15). They found that in normal individuals the coefficient of variation for TmG/GFR was 15 per cent. The coefficient of variation for TmP/GFR in the normal subjects included in Table II is 30 per cent.

DISCUSSION

Our data are in accord with the observations in the dog (1–5) and in man (6) which indicate that there is a maximal rate of phosphate reabsorption by the renal tubules. These results are in contrast to those obtained by Smith, Ollayos, and Winkler (7), Eggleton and Shuster (8), and Crawford, Gribetz, and Talbot (9), who found increased P reabsorption with increased filtered P without any apparent Tm. Furthermore, our results do not indicate that P reabsorption is conditioned by GFR at elevated plasma P levels such that a constant fraction of filtered P is reabsorbed. Were this conditioning to exist, the ratio, P reabsorbed/GFR, would remain constant. The data in Table II indicate that this ratio is quite variable.

It is apparent that there is a considerable variability in TmP in a given individual and between individuals. For the most part this variability remains unexplained. The attempt to correlate TmP with the dietary level of Ca and P in our studies has been inconclusive. A correlation between the level of phosphate in the diet and phosphate reabsorption has been demonstrated in rats (16), dogs (17) and newborn infants (18). A high level of P intake was associated with a reduction in P reabsorption. Our failure to demonstrate a clear-cut effect on TmP with an elevated P intake may be related to the fact that the amount of dietary P per kg. of body weight was lower than that achieved in rats (16) and in infants (18). That factors other than diet influence phosphate reabsorption is known. Roberts and Pitts showed that the administration of cortisone to dogs consistently reduced TmP (19). A reduction in TmP with prolonged P infusions has been shown by Michie (20) and by Hogben and Bollman (2). The latter workers showed in studies on dogs that this reduction was usually accompanied by a decrease in plasma K, and that addition of K to the infusion reversed the drop in Tm. In the occasional instance in our studies where TmP fell during an experiment there was no associated decrease in plasma K. Our experiments were usually short in comparison with those of Hogben and Bollman, and it is possible that we might have seen reductions in TmP and plasma K if the experiments had been prolonged. However, in two experiments on two normal subjects in which prolonged P infusions were given no decrease in TmP was noted. Clearly, our data do not reveal the cause of fluctuations in TmP. Elucidation of these factors will require further experimentation.

The range of values for TmP in this group of normal subjects makes the use of any average figure of little value and indicates the necessity of using the subject as his own control in any study designed to evaluate factors influencing TmP. Furthermore, the uncontrolled and unexplained "spontaneous fluctuations" in TmP make it apparent that small changes in TmP cannot be accepted as significant without critical analysis of control studies.

Certain considerations of the mechanism of phosphate reabsorption are pertinent to an evaluation of our data. The reabsorption of phosphate, like that of glucose, shows a limited maximal transfer rate. However, differences exist in the transfer characteristics of these two substances. Whereas the maximal rate of glucose transport is quite constant in any one individual and between individuals (when differences in body surface area are taken into consideration), the maximal rate of phosphate reabsorption is more variable. What is the significance of this fact? The maximal rate of glucose reabsorption is considerably

greater than is required for total reabsorption even with physiological variations in load. Thus, the kidney serves to conserve the body stores of glucose and is not normally an important regulator of plasma concentration. On the other hand, the Tm for phosphate is only slightly greater, and in some instances no greater, than reabsorption at endogenous plasma levels. Hence, relatively small changes in Tm would be expected to affect the plasma concentration of phosphate, whereas this would not be true for glucose. It may be postulated that the considerable variability of TmP is due to factors which act upon the phosphate transport mechanism to alter reabsorption of phosphate, thus providing an important mechanism for the regulation of plasma phosphate concentration. Our studies and those of others have indicated that a parathyroid hormone is one factor of importance in the regulation of phosphate transport in the renal tubule (4, 21–23).

A consideration of maximal reabsorptive and secretory rates in the renal tubules led Shannon to a general kinetic analysis for Tm substances (24). Similar kinetic considerations apply to any enzyme-catalyzed reaction where the reaction rate reaches a maximum value with increasing substrate concentration (plasma concentration) because of enzyme saturation. The maximal rate of renal tubular transport of any substance (Tm) may be visualized as that rate which is observed when the enzyme system(s) involved is saturated with regard to substrate. Since factors (*e.g.*, parathyroid hormone) other than substrate concentration may influence the activity of the enzyme system it is not surprising to find that variations in Tm values occur. It seems likely that an explanation of the variability of TmP will require an understanding of the mechanisms by which substances exert their effects on the enzymes involved in phosphate transport.

SUMMARY AND CONCLUSIONS

1. A maximal renal tubular rate of reabsorption of phosphate (TmP) has been demonstrated in normal and hypoparathyroid subjects.

2. In man considerable variability in TmP often occurs within a given experiment and also between experiments. This variability remains unexplained.

3. Dietary variations in calcium and phosphorus produced no consistent changes in TmP. Data in one subject suggested that TmP is greatest when the dietary level of P is low, and least when dietary P is high.

4. Comparison of TmP between normal individuals indicated a wide range of values.

5. The values for TmP in hypoparathyroid subjects fell within the broad range of normal. The TmP in one hyperparathyroid subject fell below the normal range.

6. Because of the variability of TmP in a given individual and between individuals each subject must serve as his own control in any study designed to determine the influence of an experimental regimen on phosphate reabsorption.

7. The possible significance of the variability of TmP is discussed in relation to current concepts of transport mechanisms.

ACKNOWLEDGMENTS

We wish to express our gratitude to Misses Mary Margaret Brown and Doris Graves for their technical assistance.

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ANNOUNCEMENT OF MEETING

The 49th Annual Meeting of the American Society for Clinical Investigation will be held in Atlantic City, New Jersey, on Monday, May 6, 1957, with headquarters at the Chalfonte-Haddon Hall. The scientific session will begin at 9 A.M. at the Steel Pier Theater.

THE EFFECTS OF PARATHYROID EXTRACT ON RENAL FUNCTION IN MAN

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The evidence that parathyroid extract acts directly on bone appears conclusive (1–3). Whether the extract exerts an effect on the kidney, however, has not been so convincingly answered. It has long been known that an increase in urinary phosphate follows the administration of parathyroid extract (4, 5), but the mechanism of the phosphaturia has not been clearly elucidated.

Although the osteoclastic action of parathyroid hormone could effect an increase in urinary inorganic phosphate, it could not simultaneously produce the hypophosphatemia observed in patients with hyperparathyroidism. Inhibition by the hormone of the renal mechanism for conserving phosphate, on the other hand, would account for both a rise in urinary and a fall in plasma phosphate.

Our studies demonstrate that a depression of the maximal renal tubular reabsorptive capacity for phosphate follows the administration of parathyroid extract, although in normal subjects prolonged treatment is required before such an effect is demonstrable.

SUBJECTS AND METHODS

Forty studies were carried out in nine of the normal subjects, the three patients with post-thyroidectomy hypoparathyroidism and the hyperparathyroid male described in the previous paper (6). The experimental procedures have been outlined (6).[3] All of the experiments were performed with the patients in the post-absorptive state.

Four lots of commercial (Eli Lilly & Company) parathyroid extract were used. Each lot was shown to be effective as demonstrated by a rise in serum Ca in at least one subject. Dosage is expressed in U.S.P. units, as assayed by the manufacturer. When given intravenously the hormone was usually administered over a period of 4 to 8 minutes. For the more prolonged studies it was given subcutaneously at 6 to 12 hourly intervals, except where otherwise indicated.

To study the effects of PTH on TmP, buffered sodium phosphate, pH 7.40, was infused until conditions of renal tubular saturation were assured. Simultaneous with the intravenous administration of PTH a sustaining infusion was begun containing, in addition to inulin and PAH, an amount of sodium phosphate calculated to maintain as constant as possible the elevated plasma P level. Thus, observations of the influence of PTH on TmP were permitted without the possible interference of rapidly changing plasma levels.

RESULTS

Acute effects of intravenous parathyroid extract at endogenous levels of plasma P

A rise in urinary phosphate occurred immediately following the intravenous administration of 200 to 1,000 units of PTH both to normal (Figure 1) and to hypoparathyroid (Figure 2) subjects. A typical study in a normal subject is outlined in Table I, while data from studies in three normal subjects are summarized in Table II. In two of the normal individuals, C. H., and J. N., the rise in urinary P occurred as a result of an increase in filtered P, which in turn, was caused by an increase in GFR. Phosphate reabsorption was increased in one subject and unchanged in the other. In the third normal individual, E. E., the increase in excreted P could be accounted for by a decrease in tubular reabsorption of P (Table II). No change in serum calcium was observed during the 2 to 4 hours following the administration of PTH. Flushing frequently followed the intravenous administration of PTH, and transient headache was an occasional complaint. A mild and brief tachycardia often occurred, but no changes in temperature or blood pressure were observed. There was

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[3] The following abbreviations will be employed: GFR, glomerular filtration rate; RPF, effective renal plasma flow; C_{In}, inulin clearance; C_{PAH}, para-amino-hippurate clearance; Tm, maximal renal tubular transport rate; PTH, parathyroid extract; Ca, calcium; P, inorganic phosphate.

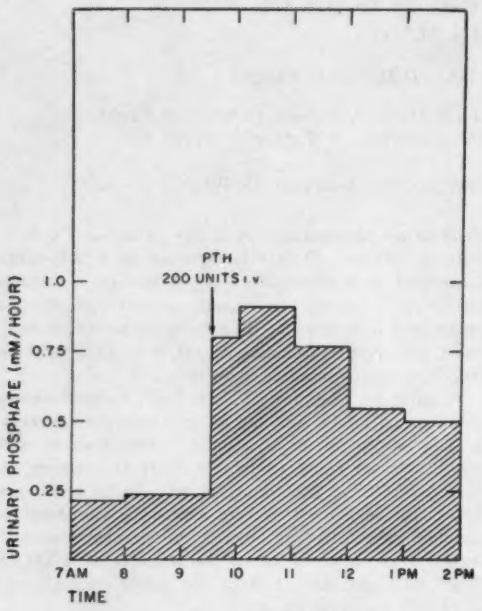

FIG. 1. URINARY PHOSPHATE EXCRETION BEFORE AND FOLLOWING THE INTRAVENOUS ADMINISTRATION OF 200 UNITS OF PARATHYROID EXTRACT TO R. H., A 24-YEAR-OLD NORMAL FEMALE

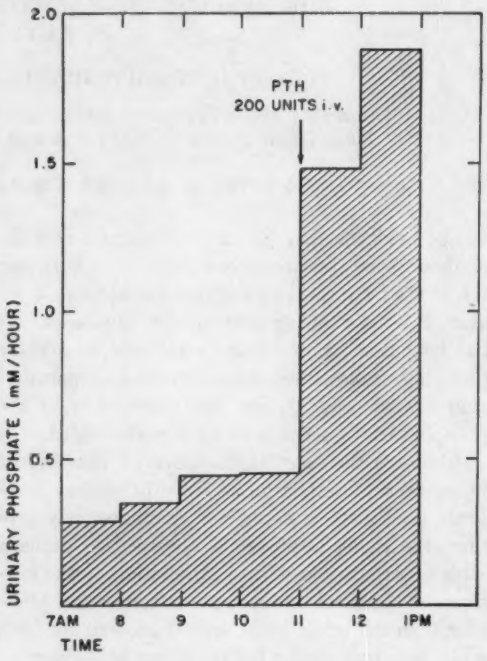

FIG. 2. URINARY PHOSPHATE EXCRETION BEFORE AND FOLLOWING THE INTRAVENOUS ADMINISTRATION OF 200 UNITS OF PARATHYROID EXTRACT TO L. D., A 52-YEAR-OLD FEMALE WITH POST-OPERATIVE HYPOPARATHYROIDISM

no evidence of sensitization or resistance, even in subjects who received several injections over a period of twelve months.

Acute effects of intravenous parathyroid extract under conditions of phosphate loading

As was noted at endogenous plasma P levels, no constant change in renal tubular reabsorption of P resulted from the administration of PTH to subjects receiving P infusions (Table III). If in the study summarized in Table III one selects two periods with comparable levels of plasma P, *e.g.*, the 6th, before the administration of PTH, and the 7th, after PTH was given, one notes that after PTH the excretion of P is increased. The rise in urinary P is ascribable to an increase in filtered P, the reabsorbed P remaining unchanged. Similar findings were noted in nine studies in five normal subjects (Table IV). The levels of TmP following the administration of PTH did not differ significantly from those seen prior to the administration of the hormone (Figure 3). D. F., who showed the greatest fall in TmP following PTH, was unique among the subjects studied in that spontaneous variations of his TmP of similar magnitude were observed on occasions when no medication was given. With but one exception a rise in GFR followed the intravenous administration of PTH, and on all occasions a striking rise in renal plasma flow was observed (Table IV). In almost all normal subjects the rise in GFR was adequate to account for the observed increase in P excretion.

In the hypoparathyroid subjects, on the other hand, the rise in urinary P which followed the administration of PTH resulted not only from an increase in filtered P, but from a striking diminution in the reabsorbed P as well (Table V). A fall in TmP was seen in all three hypoparathyroid subjects, although in A. W. a negligible decrease in response to PTH was observed on one occasion (Figure 4). The same slight but consistent rise in GFR and striking increase in RPF noted in

TABLE I

EFFECTS OF INTRAVENOUS PARATHYROID EXTRACT ON RENAL FUNCTION AT ENDOGENOUS PLASMA PHOSPHATE LEVELS

ELAPSED TIME Min.	C_{PAH} ml/min.	C_{IN} ml/min.	PLASMA P μM/ml.	PHOSPHATE		
				FILTERED μM/min.	EXCRETED μM/min.	REABSORBED μM/min.
82 – 106	535	122	1.01	123	8	115
106 – 123	518	108	1.04	113	7	106
123 – 152	458	106	1.03	109	6	103
152 – 189	838	138	1.00	138	20	118
189 – 217	825	130	0.96	125	24	101

J. N., 22 year old normal male.
Sustaining inulin infusion begun at 51 min.
155 – 160 min., 1000 USP units of parathyroid extract i.v.

TABLE II

ACUTE EFFECTS OF INTRAVENOUS PARATHYROID EXTRACT AT ENDOGENOUS PLASMA PHOSPHATE LEVELS IN NORMAL INDIVIDUALS

Subject	Units of Extract	C_{PAH} ml/min.		C_{IN} ml/min.		Plasma P μM/ml		Phosphate μM/min.					
								Filtered		Excreted		Reabsorbed	
		Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
C.H.	800	550	674	121	145	1.12	1.27	136	184	2	15	134	169
E.E.	800	620	930	130	131	1.08	1.06	140	139	3	18	137	121
J.N.	1000	502	832	112	134	1.03	0.98	115	131	7	22	108	109

TABLE III

EFFECTS OF INTRAVENOUS PARATHYROID EXTRACT ON RENAL FUNCTION DURING PHOSPHATE LOADING

Period	Elapsed Time min.	Urine Flow ml/min.	C_{PAH} ml/min.	C_{IN} ml/min.	Plasma P μM/ml	Phosphate μM/min.		
						Filtered	Excreted	Reabsorbed
	0	Inulin, PAH infusion begun.						
1	41 – 59	5.45	538	142	1.09	155	4	151
2	59 – 75	8.00	500	135	1.07	144	4	140
	76	Inulin, PAH, buffered sodium phosphate infusion begun.						
3	109 – 139	8.90	543	140	2.16	302	92	210
4	139 – 170	5.00	502	142	2.52	358	152	206
5	201 – 221	4.85	463	130	3.53	459	262	197
6	221 – 243	3.18	500	141	4.02	566	329	237
	244	Maintenance inulin, PAH, phosphate infusion begun.						
	247 – 251	200 USP units of parathyroid extract i.v.						
7	279 – 301	3.45	652	152	4.15	625	386	239
8	301 – 319	2.56	591	144	4.10	590	348	242

C.H. 17-year old normal male.

TABLE IV

ACUTE EFFECTS OF INTRAVENOUS PARATHYROID EXTRACT ON RENAL FUNCTION IN NORMAL SUBJECTS

Date	Subject	Units of Extract	C_{PAH}			C_{IN}			TmP		
			Before ml/min.	After ml/min.	Percent Change	Before ml/min.	After ml/min.	Percent Change	Before μM/min.	After μM/min.	Percent Change
3/25/54	C. H.	200	602	670	+ 11	149	149	0	190	215	+ 13
4/ 6/54	C. H.	200	500	620	+ 24	138	148	+ 7	217	241	+ 11
7/13/54	C. H.	500	763	865	+ 13	151	164	+ 9	138	145	+ 5
9/ 7/54	C. H.	500	561	810	+ 44	135	148	+ 10	239	204	- 15
5/25/54	E. E.	900	627	1048	+ 67	135	153	+ 13	169	208	+ 23
6/10/54	J. N.	1000	475	725	+ 53	106	118	+ 11	177	157	- 11
6/22/54	J. N.	1000				124	137	+ 10	176	185	+ 5
3/11/55	D. F.	500	387	620	+ 60	91	99	+ 9	82	59	- 28
3/31/55	E. P.	500	537	741	+ 38	96	118	+ 23	99	127	+ 28

normal subjects occurred in the hypoparathyroid patients (Table VI).

The acute administration of PTH had no discernible immediate effect on plasma calcium or potassium or on urinary calcium in any of the subjects.

Effects of prolonged treatment with parathyroid extract

Hyperparathyroidism was induced in five normal subjects on six occasions and in two hypoparathyroid patients by the administration of 1,800 to 4,500 units of extract over periods of 60 to 120 hours. In all subjects hypercalcemia, hypophosphatemia, and an increase in urinary calcium and phosphate were observed (Table VII). Two subjects complained of mild skeletal pain, anorexia, polydipsia, polyuria, and constipation.

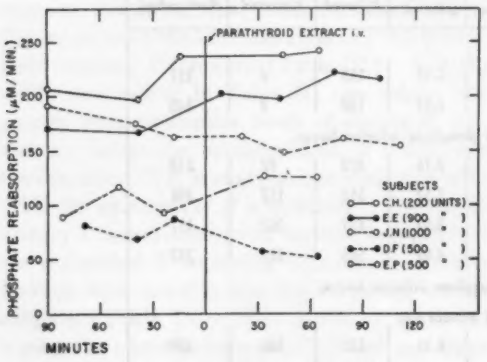

FIG. 3. VARIATIONS IN TmP IN FIVE NORMAL SUBJECTS BEFORE AND FOLLOWING THE INTRAVENOUS ADMINISTRATION OF PARATHYROID EXTRACT

These symptoms, as well as the chemical abnormalities rapidly disappeared after discontinuation of the hormone.

The hyperparathyroid state was characterized by a depression of TmP in both normal (Table VIII) and hypoparathyroid (Table IX) subjects. The fall in TmP ranged from 27 to 64 per cent below control levels (Table X). In W. R. on one occasion (5/6/55) the TmP was reduced by parathyroid extract to the level observed in the only patient with spontaneous hyperparathyroidism studied. The latter, a 72-year-old male with a plasma Ca of 14.05 mg. per 100 ml. and a plasma P of 0.53 μM per ml. was found to have a TmP of 26 μM per minute.

The effect of the prolonged administration of parathyroid extract on C_{In} and on C_{PAH}, in contrast to the results in the acute studies, was inconstant (Table X).

Renal function studies were carried out in a hypoparathyroid patient in the untreated state (plasma Ca, 7.10 mg. per cent; P, 1.42 μM per ml.), in an "isoparathyroid" state, following the daily administration of 100 units of extract for 5 days (plasma Ca, 10.05; P, 1.10), and in a hyperparathyroid state, following the daily administration of 900 units of extract for 3 days (plasma Ca, 12.90; P, 0.68). Average TmP values of 170, 150, and 70 μM per minute, respectively, were found at these times (Figure 5). In a similar series of studies in a second hypoparathyroid subject, B. C., a progressive lowering of TmP with increasing doses of extract was again noted (Table XI). Of interest is the fact that 100 units of parathyroid extract daily for 3 days served to

TABLE V

ACUTE EFFECTS OF PARATHYROID EXTRACT ON RENAL FUNCTION IN HYPOPARATHYROIDISM

ELAPSED TIME Min.	C_{PAH} ml/min.	C_{IN} ml/min.	PLASMA P μM/ml	PHOSPHATE		
				FILTERED μM/min.	EXCRETED μM/min.	REABSORBED μM/min.
77 – 97	345	80.0	2.26	181	79	102
97 – 116	316	73.0	2.60	190	96	94
116 – 136	315	75.5	3.00	226	116	110
159 – 192	711	84.0	2.80	235	177	58
192 – 216	625	90.0	2.57	231	183	48

L. D., 52 year old female, post-operative hypoparathyroidism.
Continuous infusion of buffered sodium phosphate and inulin.
137 – 145 min., Parathyroid extract, (Lilly no. 628857), 500 USP units i.v.

lower B. C.'s plasma P and TmP without altering the level of plasma Ca. Hyperparathyroidism was induced in this subject by intermittent intravenous administration of extract.[4] This was the only one of the prolonged studies reported in hypoparathyroid patients in which PTH was given by other than the subcutaneous route. D. F. and W. R., two normal subjects, received three successive daily infusions, each containing 1,500 units of extract, and each given intravenously over a six-hour period. No effect on plasma Ca or P or on TmP (measured 15 hours after the last infusion of PTH) was observed. In W. R., an equal quantity of the same lot of hormone given in repeated *subcutaneous* injections over a similar period produced marked hyperparathyroidism (Table VII).

DISCUSSION

Two explanations have been offered for the increase in urinary phosphate usually observed following the administration of parathyroid extract. The evidence supporting these hypotheses, an increase in the load of phosphate filtered through the kidney, on the one hand, and a diminution in the tubular reabsorption of phosphate, on the other, has been summarized by Bartter (8). The rise in filtered phosphate has been ascribed to an increase in glomerular filtration rate, to a rise in plasma phosphate, or to both (9–11). Indeed, the demonstration that phosphaturia follows the administration to animals of formalin-inactivated (as assayed by its effect on serum calcium) parathyroid extract has been interpreted as indicating that the observed increase in urinary phosphate is an artifact ascribable to some non-hormonal component of an exceedingly heterogeneous preparation (12).

Our data indicate that the phosphaturia seen immediately following the intravenous administration of the extract to normal individuals is the result of an increase in GFR with a consequent

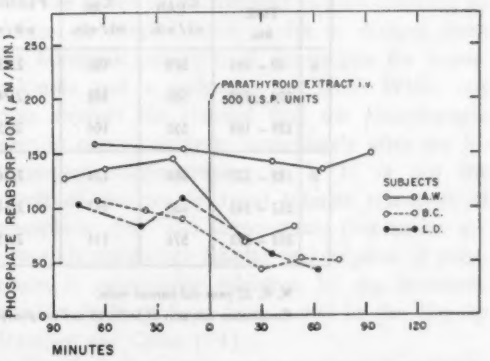

FIG. 4. CHANGES IN TmP FOLLOWING THE ADMINISTRATION OF PARATHYROID EXTRACT TO THREE SUBJECTS WITH HYPOPARATHYROIDISM

[4] The fall in C_{IN} in this subject in the hyperparathyroid state was believed ascribable to mild congestive heart failure, a state known to be accompanied by a reduced GFR (7). Digitalis, with which she had been treated for signs of failure noted one year previously, had been omitted prior to the administration of parathyroid extract.

TABLE IV

ACUTE EFFECTS OF INTRAVENOUS PARATHYROID EXTRACT ON RENAL FUNCTION IN NORMAL SUBJECTS

Date	Subject	Units of Extract	C_{PAH}			C_{IN}			TmP		
			Before ml/min.	After ml/min.	Percent Change	Before ml/min.	After ml/min.	Percent Change	Before μM/min.	After μM/min.	Percent Change
3/25/54	C. H.	200	602	670	+ 11	149	149	0	190	215	+ 13
4/ 6/54	C. H.	200	500	620	+ 24	138	148	+ 7	217	241	+ 11
7/13/54	C. H.	500	763	865	+ 13	151	164	+ 9	138	145	+ 5
9/ 7/54	C. H.	500	561	810	+ 44	135	148	+ 10	239	204	- 15
5/25/54	E. E.	900	627	1048	+ 67	135	153	+ 13	169	208	+ 23
6/10/54	J. N.	1000	475	725	+ 53	106	118	+ 11	177	157	- 11
6/22/54	J. N.	1000				124	137	+ 10	176	185	+ 5
3/11/55	D. F.	500	387	620	+ 60	91	99	+ 9	82	59	- 28
3/31/55	E. P.	500	537	741	+ 38	96	118	+ 23	99	127	+ 28

normal subjects occurred in the hypoparathyroid patients (Table VI).

The acute administration of PTH had no discernible immediate effect on plasma calcium or potassium or on urinary calcium in any of the subjects.

Effects of prolonged treatment with parathyroid extract

Hyperparathyroidism was induced in five normal subjects on six occasions and in two hypoparathyroid patients by the administration of 1,800 to 4,500 units of extract over periods of 60 to 120 hours. In all subjects hypercalcemia, hypophosphatemia, and an increase in urinary calcium and phosphate were observed (Table VII). Two subjects complained of mild skeletal pain, anorexia, polydipsia, polyuria, and constipation.

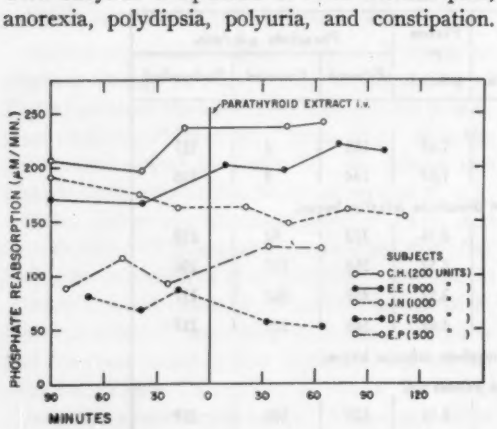

FIG. 3. VARIATIONS IN TmP IN FIVE NORMAL SUBJECTS BEFORE AND FOLLOWING THE INTRAVENOUS ADMINISTRATION OF PARATHYROID EXTRACT

These symptoms, as well as the chemical abnormalities rapidly disappeared after discontinuation of the hormone.

The hyperparathyroid state was characterized by a depression of TmP in both normal (Table VIII) and hypoparathyroid (Table IX) subjects. The fall in TmP ranged from 27 to 64 per cent below control levels (Table X). In W. R. on one occasion (5/6/55) the TmP was reduced by parathyroid extract to the level observed in the only patient with spontaneous hyperparathyroidism studied. The latter, a 72-year-old male with a plasma Ca of 14.05 mg. per 100 ml. and a plasma P of 0.53 μM per ml. was found to have a TmP of 26 μM per minute.

The effect of the prolonged administration of parathyroid extract on C_{In} and on C_{PAH}, in contrast to the results in the acute studies, was inconstant (Table X).

Renal function studies were carried out in a hypoparathyroid patient in the untreated state (plasma Ca, 7.10 mg. per cent; P, 1.42 μM per ml.), in an "isoparathyroid" state, following the daily administration of 100 units of extract for 5 days (plasma Ca, 10.05; P, 1.10), and in a hyperparathyroid state, following the daily administration of 900 units of extract for 3 days (plasma Ca, 12.90; P, 0.68). Average TmP values of 170, 150, and 70 μM per minute, respectively, were found at these times (Figure 5). In a similar series of studies in a second hypoparathyroid subject, B. C., a progressive lowering of TmP with increasing doses of extract was again noted (Table XI). Of interest is the fact that 100 units of parathyroid extract daily for 3 days served to

TABLE V

ACUTE EFFECTS OF PARATHYROID EXTRACT ON RENAL FUNCTION IN HYPOPARATHYROIDISM

ELAPSED TIME Min.	C_{PAH} ml/min.	C_{IN} ml/min.	PLASMA P μM/ml	PHOSPHATE		
				FILTERED μM/min.	EXCRETED μM/min.	REABSORBED μM/min.
77–97	345	80.0	2.26	181	79	102
97–116	316	73.0	2.60	190	96	94
116–136	315	75.5	3.00	226	116	110
159–192	711	84.0	2.80	235	177	58
192–216	625	90.0	2.57	231	183	48

L. D., 52 year old female, post-operative hypoparathyroidism.
Continuous infusion of buffered sodium phosphate and inulin.

137 – 145 min., Parathyroid extract, (Lilly no. 628857), 500 USP units i.v.

lower B. C.'s plasma P and TmP without altering the level of plasma Ca. Hyperparathyroidism was induced in this subject by intermittent intravenous administration of extract.[4] This was the only one of the prolonged studies reported in hypoparathyroid patients in which PTH was given by other than the subcutaneous route. D. F. and W. R., two normal subjects, received three successive daily infusions, each containing 1,500 units of extract, and each given intravenously over a six-hour period. No effect on plasma Ca or P or on TmP (measured 15 hours after the last infusion of PTH) was observed. In W. R., an equal quantity of the same lot of hormone given in repeated *subcutaneous* injections over a similar period produced marked hyperparathyroidism (Table VII).

DISCUSSION

Two explanations have been offered for the increase in urinary phosphate usually observed following the administration of parathyroid extract. The evidence supporting these hypotheses, an increase in the load of phosphate filtered through the kidney, on the one hand, and a diminution in the tubular reabsorption of phosphate, on the other, has been summarized by Bartter (8). The rise in filtered phosphate has been ascribed to an increase in glomerular filtration rate, to a rise in plasma phosphate, or to both (9–11). Indeed, the demonstration that phosphaturia follows the administration to animals of formalin-inactivated (as assayed by its effect on serum calcium) parathyroid extract has been interpreted as indicating that the observed increase in urinary phosphate is an artifact ascribable to some non-hormonal component of an exceedingly heterogeneous preparation (12).

Our data indicate that the phosphaturia seen immediately following the intravenous administration of the extract to normal individuals is the result of an increase in GFR with a consequent

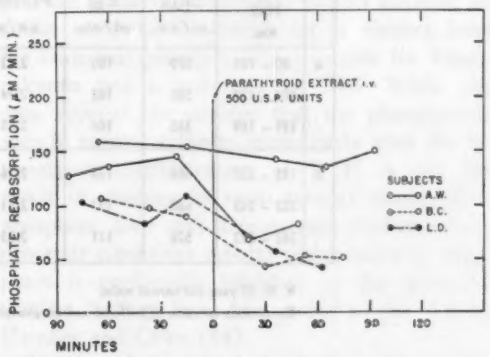

FIG. 4. CHANGES IN TmP FOLLOWING THE ADMINISTRATION OF PARATHYROID EXTRACT TO THREE SUBJECTS WITH HYPOPARATHYROIDISM

[4] The fall in C_{In} in this subject in the hyperparathyroid state was believed ascribable to mild congestive heart failure, a state known to be accompanied by a reduced GFR (7). Digitalis, with which she had been treated for signs of failure noted one year previously, had been omitted prior to the administration of parathyroid extract.

TABLE VI

ACUTE EFFECTS OF INTRAVENOUS PARATHYROID EXTRACT ON RENAL FUNCTION IN HYPOPARATHYROIDISM

Date	Subject	Units of Extract	C_{PAH}			C_{IN}			T_mP		
			Before ml/min.	After ml/min.	Percent Change	Before ml/min.	After ml/min.	Percent Change	Before μM/min.	After μM/min.	Percent Change
8/25/54	A. W.	500				96	103	+ 7	158	142	- 10
2/25/55	A. W.	500	498	852	+ 71	108	113	+ 5	137	75	- 45
2/22/55	B. C.	500	230	418	+ 75	63	69	+ 10	98	50	- 49
3/9/55	L. D.	500	325	668	+ 102	75	87	+ 16	99	51	- 48

TABLE VII

EFFECTS OF PROLONGED ADMINISTRATION OF PARATHYROID EXTRACT ON PLASMA AND URINARY CALCIUM AND PHOSPHORUS

Subject	Total Units of Extract	Hours of Administration	Plasma Ca mg. percent		Plasma P μM/ml		Urine Ca μgm/min.		Urine P μM/min.	
			Before	After	Before	After	Before	After	Before	After
Normal										
C. H. 7/13/54	4000	60	9.80	13.5[illegible]	[illegible]23	1.02			9	52
W. R. 8/19/54	4500	72	10.02	13.90	0.96	0.73			23	52
W. R. 5/6/55	4000	72	9.40	15.20	0.98	0.63	280	420	7	27
E. P. 3/31/55	2500	120	10.60	12.90	1.17	0.68	213	506	8	20
L. B. 11/22/54	2100	72	9.20	12.00	0.90	0.70	257	444	16	24
W. H. 10/22/54	1800	66	8.60	11.31	1.23	0.96	161	212	9	25
Hypoparathyroid										
A. W. 9/30/54	3000	96	7.10	12.90	1.42	0.68	35	90	0.9	21
B. C. 2/24/54	2500	60	8.08	11.95	1.35	0.74				

TABLE VIII

RENAL EXCRETION OF PHOSPHATE BEFORE (A) AND AFTER (B) ADMINISTRATION OF 1500 USP UNITS OF PARATHYROID EXTRACT DAILY FOR 3 DAYS

	ELAPSED TIME Min.	C_{PAH} ml/min.	C_{IN} ml/min.	PLASMA P μM/ml.	PHOSPHATE		
					FILTERED μM/min.	EXCRETED μM/min.	REABSORBED μM/min.
A	80 – 109	570	100	2.43	243	104	139
	109 – 139	580	102	2.74	279	143	136
	139 – 169	555	106	3.05	324	181	143
B	183 – 222	684	124	2.36	292	214	78
	222 – 243	665	107	2.61	279	225	54
	266 – 293	576	111	2.33	259	213	46

W. R. 22 year old normal male.
Continuous infusion of buffered sodium phosphate and inulin.

rise in filtered phosphate. In hypoparathyroid subjects the relative increase in phosphate excretion is even more striking, for in such individuals parathyroid extract produces not only a rise in filtration rate, but a depression of tubular reabsorption of phosphate as well. Repeated subcu-

TABLE IX

RENAL EXCRETION OF PHOSPHATE BEFORE (A) AND AFTER (B) ADMINISTRATION OF 900 USP UNITS OF PARATHYROID EXTRACT DAILY FOR 3 DAYS

	ELAPSED TIME Min.	C_{PAH} ml/min.	C_{IN} ml/min.	PLASMA P μM/ml.	PHOSPHATE		
					FILTERED μM/min.	EXCRETED μM/min.	REABSORBED μM/min.
A	168 – 205	86	86.5	2.65	229	60	169
	205 – 232	95	86.0	3.17	272	98	174
	265 – 296	95	82.5	4.36	360	188	172
B	165 – 195	206	131	1.77	232	163	69
	228 – 256	218	133	2.35	312	267	45
	256 – 286	212	129	3.07	396	295	101

A. W., 37 year old hypoparathyroid male.
Continuous infusion of inulin and buffered sodium phosphate.

TABLE X

EFFECTS OF PROLONGED ADMINISTRATION OF PARATHYROID EXTRACT ON RENAL FUNCTION

Subject	Total Units of Extract	Hours of Administration	C_{PAH}			C_{IN}			TmP		
			Before ml/min.	After ml/min.	Percent Change	Before ml/min.	After ml/min.	Percent Change	Before μM/min.	After μM/min.	Percent Change
Normal											
C. H. 7/13/54	4000	60	550	763	+ 39	135	151	+ 12	200	138	- 31
W. R. 8/19/54	4500	72	585	728	+ 24	113	121	+ 7	122	63	- 48
W. R. 5/ 6/55	4000	72	558	568	+ 2	102	95	- 7	81	29	- 64
E. P. 3/31/55	2500	120	537	430	- 20	96	85	- 11	110	64	- 42
L. B. 11/22/54	2100	72	846	837	- 1	136	131	- 4	124	91	- 27
W. H. 10/22/54	1800	66	904	889	- 2	125	129	+ 3	179	118	- 34
Hypoparathyroid											
A. W. 9/30/54	2700	72	157	200	+ 27	96	129	+ 34	175	72	- 59
B. C. 2/24/54	2500	60	236	232	- 2	74	57	- 23	131	56	- 57

taneous administration of extract, however, both to normal and to hypoparathyroid subjects induces a state of hyperparathyroidism, characterized by an invariable and striking diminution in TmP. No effort was made to determine the minimal period of extract administration required before a tubular effect is demonstrable in normal individuals; sixty hours was the shortest duration of an experiment involving the subcutaneous administration of hormone, and a 31 per cent decrease in TmP was observed. Why the tubular effect is more readily demonstrable in the hypoparathyroid than in the normal individual is unknown, but this phenomenon may have a parallel in the increased sensitivity to small amounts of thyroid noted in patients with myxedema (13).

Of considerable interest is the fact that the induced hyperparathyroid state in several of our subjects was not accompanied by a rise in GFR or in RPF. These observations strengthen the thesis that the factor in parathyroid extract exerting an action on renal hemodynamics is distinct from the hormonal principle(s) responsible for hypercalcemia and a reduction in TmP. While our data support the concept that the phosphaturia seen in normal subjects immediately after the intravenous administration of PTH is not the result of changes in renal tubular transport of phosphate, they also demonstrate that under appropriate conditions tubular reabsorption of phosphate is profoundly inhibited by the hormone. Similar findings have been noted in the dog by Handler and Cohn (14).

The fact that prolonged administration of the extract is required to depress TmP in normal individuals raises the question as to whether the

TABLE XI

EFFECTS OF PARATHYROID EXTRACT IN HYPOPARATHYROIDISM (B. C.)

Date	Parathyroid Extract	Time Elapsed min.	Urine Flow ml/min.	Plasma Ca mg %	Plasma P μM/ml	C_{PAH} ml/min.	C_{IN} ml/min.	Phosphate (μM/min.) Filtered	Excreted	Reabsorbed
2/9/55	None	0		8.08	1.35					
		26 Continuous infusion of inulin, PAH, and phosphate								
		89 – 121	6.85	7.40	3.33	252	75.5	252	114	138
		121 – 153	8.07		3.73	234	73.6	274	143	131
		153 – 190	7.81	5.36	4.00	222	71.5	286	161	125
		Mean				236	73.5	271	139	132
2/14/55	50 u. bid for 3 days	0		7.56	0.91					
		26 Continuous infusion of inulin, PAH, and phosphate								
		93 – 124	8.32	7.09	2.67	266	84.0	224	131	93
		124 – 157	8.82		2.94	265	82.9	244	166	78
		157 – 187	9.40	6.83	3.32	258	83.3	277	194	83
		Mean				259	83.4	248	164	85
2/24/55	500 u. q. 12 hr. x 5 i. v.	0		11.95	0.74					
		109 Continuous infusion of inulin, PAH, and phosphate								
		173 – 205	3.41		2.57	220	57.7	148	98	50
		205 – 241	3.65		3.02	225	55.5	168	113	55
		241 – 270	4.01	9.03	3.52	252	56.5	199	137	62
		Mean				232	56.6	172	116	56

hormone acts directly on the renal tubule. That the hypercalcemia produced by the hormone is not responsible for the change in tubular transport of P is indicated by the demonstration that a rise in plasma Ca leads to an increase in the tubular reabsorption of P (15). In addition, the prompt and striking fall in TmP which follows the intravenous administration of parathyroid extract to hypoparathyroid subjects strongly suggests a direct effect of the hormone on the renal tubule.

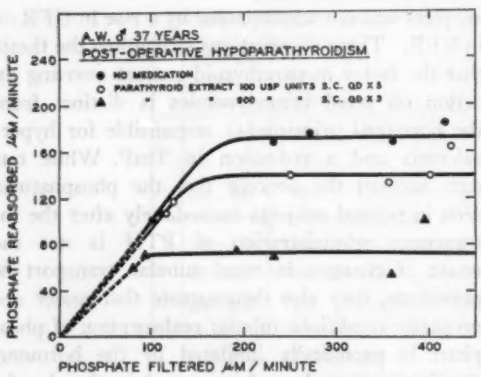

FIG. 5. TUBULAR REABSORPTION OF PHOSPHATE AT SEVERAL LEVELS OF FILTERED PHOSPHATE IN A HYPOPARATHYROID SUBJECT IN THE UNTREATED STATE, IN AN "ISOPARATHYROID" STATE, AND IN A HYPERPARATHYROID STATE

No manifestations of hyperparathyroidism were observed in W. R. following the intravenous administration of 1500 units of extract over a six-hour period on each of three successive days. Whether the renal tubule and bone of the normal individual must be constantly exposed to the extract for an even more prolonged period of time before a change in TmP or in calcium metabolism is demonstrable remains to be answered.

The demonstration of an action of PTH on the kidney does not, of course, exclude an effect on bone. Indeed, only by invoking an action of the hormone at both sites can one satisfactorily explain the chemical aberrations characteristic of patients with hyperparathyroidism. It would appear reasonable, then, to assume that the secretion(s) of the parathyroid alters metabolic processes in bone, in kidney, and possibly even elsewhere.

SUMMARY

1. The intravenous administration of parathyroid extract results in a phosphate diuresis. Such an effect in normal subjects can generally be accounted for by a rise in filtered phosphate secondary to a rise in glomerular filtration rate with-

out any decrease in phosphate Tm. In hypoparathyroid individuals there is a diminution in the tubular reabsorption of phosphate as well as an increase in filtered phosphate.

2. Repeated subcutaneous injections of parathyroid extract both to normal and to hypoparathyroid individuals lead to a diminution in the maximal tubular reabsorptive capacity for phosphate.

3. The phosphaturia seen after the *acute* administration of extract to normal individuals is usually caused by an action of the extract on renal hemodynamics and is not accompanied by hypercalcemia. Following the prolonged administration of parathyroid extract to normal individuals, however, hypercalcemia is observed as well as a decrease in TmP, but without any constant change in renal hemodynamics.

ACKNOWLEDGMENT

We wish to express our gratitude to Misses Mary Margaret Brown and Doris Graves for their technical assistance.

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EFFECTS OF PHOSPHATE LOADING AND DEPLETION ON THE RENAL EXCRETION AND REABSORPTION OF INORGANIC PHOSPHATE

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A number of studies have indicated that parathyroid extract increases the renal excretion of phosphate (1–6). This effect appears to result from a depression of the renal reabsorption of phosphate (6). Endogenous secretory activity of the parathyroid glands has been related to the extracellular concentration and/or the dietary intake of calcium and phosphate. Although dietary variations of these ions did not correlate consistently with TmP [3] in our initial investigations (7), other workers have demonstrated an influence of diet on phosphate reabsorption. A high phosphate diet led to a diminished P reabsorption in normal but not in parathyroidectomized rats (8). Studies in dogs (9) and in normal human subjects (8, 10) also indicated that the level of P intake influenced P reabsorption, but whether this effect was mediated by the parathyroid glands is not clear. In order to examine more rigorously the effects of a high P intake, buffered sodium phosphate solutions were administered intravenously daily for four to eight days to the normal and hypoparathyroid subjects previously described (7). Another phase of this study involved an inquiry into the mechanism by which aluminum gels produce a marked reduction in urinary P.

Variations in plasma levels of Ca and P often occur together. An attempt was made to separate the effects of elevation of plasma P from the frequently seen simultaneous reduction in plasma Ca.

Our data indicate that repeated daily intravenous administration of large quantities of phosphate results in a reduction in renal P reabsorption. This effect does not appear to depend upon an associated reduction in plasma Ca. Whether this effect is mediated via the parathyroids cannot be conclusively stated. The results indicate that aluminum gels produce their effect primarily, and perhaps solely, by effecting a reduction in plasma P.

METHODS

These studies were conducted on the same patients previously described, and the measurements of TmP were carried out by the method detailed in an earlier paper (7). In studies of the effects of intravenously administered phosphate, a buffered sodium phosphate solution, pH 7.4, was infused over a 4 to 6-hour period on four consecutive days. Five subjects received 75 mM of P each day; two subjects, C. H. and D. F., received 90 and 150 mM P per day, respectively. Two of the normal subjects, C. H. and E. P., received eight daily P infusions, and on the last four days they received, in addition, infusions of Ca. The Ca was administered as calcium gluconate, 15 mg. Ca per kg. of body weight in 1,000 ml. of 5 per cent glucose in water, over a four-hour period starting several hours after the completion of the phosphate infusion. Infusions of di-sodium ethylenediamine tetraacetic acid (EDTA) were given to the four normal subjects who received intravenous phosphate. Four gm. of EDTA was administered in 1,000 ml. of 5 per cent glucose in water over a 4 to 6-hour period daily for four consecutive days. TmP was measured prior to, and on the day following completion of the P or EDTA infusions.

In the studies on the effects of aluminum gels 180 ml. of basic aluminum carbonate (Basaljel®) was administered orally each day in four divided doses, one hour after meals and at bedtime, for 6 to 21 days. Concomitantly, the subjects received a low dietary P intake of 600 to 800 mg. per day. Balance studies carried out during aluminum gel administration were performed on the metabolic ward in the manner described by Deitrick, Whedon, and Shorr (11).

Chemical methods and calculations used in these studies were the same as those described in a previous communication (7).

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[3] As in the previous papers, the following abbreviations have been used: Ca, calcium; P, inorganic phosphate; Tm, maximum renal tubular rate of transfer.

TABLE I

Effect of intravenous sodium phosphate on the maximal phosphate reabsorption in a normal male before (A) and after (B) four daily infusions each containing 150 mM P

Total concurrent time *min.*	Urine flow *ml./min.*	C_{Is} *ml./min.*	C_{PAH} *ml./min.*	Plasma P *μM/ml.*	Urine P *μM/ml.*	Phosphate		
						Filtered *μM/min.*	Excreted *μM/min.*	Reabsorbed *μM/min.*
			A. Control					
0	14.5			1.01	1.55		22	
71	Intravenous buffered sodium phosphate delivered at 0.5 mM/min.							
129–159	12.4	119	500	2.76	17.2	328	214	114
159–189	11.2	110	495	3.11	22.2	342	249	93
189–219	10.1	116	517	3.31	28.2	384	285	99
			B. After 4 daily phosphate infusions—150 mM/day					
0	15.3			0.59	3.86		59	
45	Phosphate infusion at 0.5 mM/min.							
103–135	9.5	99	516	1.97	21.4	195	203	−8
135–173	9.1	105	509	2.48	28.9	260	264	−4
173–202	10.7	107	525	2.82	27.4	302	293	9

TABLE II

Summary of effects of phosphate infusions on normal and hypoparathyroid subjects

		TmP		Plasma* P	Plasma* Ca	Urine* P
		μM/min.	*% control*	*μM/ml.*	*mg. %*	*μM/min.*
Normals						
C. H.	Before P	256	100	1.42	9.2	17
	After P	197	77	1.26		32
	After P and Ca†	168	66	1.08	10.20	25
D. F.	Before P	102	100	1.01	9.9	22
	After P	0	0	0.59	10.1	59
W. R.	Before P	137	100	0.97	10.30	12
	After P	59	43	0.83	9.24	30
E. P.	Before P	110	100	1.09	11.20	9
	After P	70	64	1.06	10.20	24
	After P and Ca†	45	41	0.95	8.86	29
Hypoparathyroid						
A. W.	Before P	149	100	1.37	7.10	10
	After P	134	90	1.50	5.67	3
B. C.	Before P	84	100	0.91	7.6	5
	After P	64	76	0.87	7.6	3
L. D.	Before P	102	100	0.79	8.89	0.8
	After P	40	39	0.74	8.35	5.0

* Specimens obtained before the first daily P infusion and the day following the last P infusion and prior to inulin-phosphate infusions for determination of TmP.

† Four additional daily phosphate infusions were given together with 4 daily infusions of calcium gluconate (15 mg. Ca per kg.) given over a 4 to 6 hour period several hours after the phosphate infusions had been completed.

RESULTS

Effects of intravenous phosphate in normal subjects

The administration of four daily intravenous infusions of P to one normal subject led to a fall in P reabsorption from an average of 102 μM per min. to 0 (Table I). A significant reduction in TmP was also noted in three other normal subjects (Table II). It was found that the administration of Ca infusions to subjects receiving P in-

TABLE III

Effect of EDTA infusions on plasma concentrations and urinary excretion of Ca and P

			Plasma Ca mg. %		Plasma P μM/ml.		Urine Ca mg./24 hr.	Urine P mM/24 hr.
			Before	After	Before	After		
Normals								
D. F.	3/3–4	4 gm. di-Na EDTA	9.79	9.15	1.07	0.87	353	
	3/4–5	4 gm. di-Na EDTA	8.79	8.85	0.89	0.69	338	
	3/5–6	4 gm. di-Na EDTA	9.55	8.78	1.01	0.89	370	
	3/6–7	4 gm. di-Na EDTA	10.35	9.40	1.12	0.76	280	
E. P.	4/10–11	Control					62	18.2
	4/11–12	4 gm. di-Na EDTA	11.00	9.60	1.12	0.92	356	20.0
	4/12–13	4 gm. di-Na EDTA	10.90	9.30	1.02	0.86	364	20.1
	4/13–14	4 gm. di-Na EDTA	10.30	9.20	1.11	0.87	298	16.8
	4/14–15	4 gm. di-Na EDTA			1.17	0.72	350	16.3
	Diet: 135 mg. Ca; 570 mg. P daily							
W. R.	11/15–16	4 gm. di-Na EDTA	9.58	8.57			413	46.0
	11/16–17	4 gm. di-Na EDTA	9.03	6.48	0.89	0.77	392	33.0
	11/17–18	2 gm. di-Na EDTA					189	26.0
	11/18–19	4 gm. di-Na EDTA					294	29.0
	Diet: 130 mg. Ca; 1,200 mg. P daily							
C. H.	4/14–15	4 gm. di-Na EDTA	8.45	7.55	1.25	1.24		
	4/15–16	4 gm. di-Na EDTA						
	4/16–17	4 gm. di-Na EDTA						
	4/17–18	4 gm. di-Na EDTA						
	4/18–19	4 gm. di-Na EDTA	9.45	8.20	1.29	1.47		

fusions did not reverse the downward trend in TmP (Table II).

A decrease in endogenous plasma P levels as a consequence of P infusions was seen in the normal subjects, but there was no consistent change in plasma Ca (Table II). It was noted that the endogenous urinary excretion of phosphate increased in the face of a decreasing plasma P level, a finding consistent with a reduced tubular P reabsorption.

Effects of phosphate infusions in hypoparathyroid subjects

The results of P infusions in three hypoparathyroid subjects are shown in Table II. In A. W., the most severely hypoparathyroid subject, the reduction in TmP was less than that seen in normal individuals. However, the other two hypoparathyroid subjects, whose disease was considerably milder, showed reductions in TmP as great as those seen in the normal subjects. It can be seen that the endogenous plasma P levels and P excretion changed very little in the hypoparathyroid individuals.

TABLE IV

Effect of EDTA on TmP in four normal subjects

Subject	Control TmP μM/min.	After EDTA	
		μM/min.	% Control
C. H.	256	247	97
D. F.	102	118	116
W. R.	137	146	107
E. P.	110	112	102

Effects of EDTA infusions in normal subjects

Because an elevation of plasma P is usually accompanied by a reduction of plasma Ca one must consider the possibility that the effects observed when the plasma P is high may actually result from a lowered plasma Ca. The administration of di-sodium ethylenediamine tetraacetic acid (EDTA), permitted a reduction in the circulating Ca, particularly the ionized fraction, without a concomitant increase in plasma P. The effects of intravenous EDTA administration are summarized in Table III. The fall in plasma calcium seen following an infusion of EDTA actually represents a reduction in the oxalate-precipitable, and, presumably, in the ionized calcium. The urinary excretion of Ca is considerably elevated by the administration of EDTA. It can also be seen that

a drop in plasma P usually occurred following EDTA administration. That this result is not explained by an augmented urinary P excretion is apparent from the excretion data on E. P. No effect of EDTA on TmP was noted in four normal subjects (Table IV).

Administration of aluminum gel

Oral administration of aluminum gel produced a reduction in fasting plasma P levels and in urinary excretion of P in normal individuals and in the one hypoparathyroid subject studied (Table V). There was a profound drop in plasma P in the hypoparathyroid subject, A. W., on two occasions and in one normal individual, E. E. Why the latter reacted in this fashion on only one occasion is not clear; the daily dose of gel and the dietary P level were similar on another occasion when a much less profound fall in plasma P occurred. The fall in plasma P in A. W. as a result of aluminum gel regimen was not altered by the daily administration of as much as 400 units of parathyroid extract. Calcium, phosphorus, and nitrogen balance studies were carried out in the hypoparathyroid subject and in one normal individual before and during a course of aluminum gel. It can be seen (Figure 1 and Figure 2) that the administration of the gel resulted in an essentially P-free urine. A negative P balance was observed in both subjects but was much greater in the hypoparathyroid individual, and could be accounted for by an increased fecal P. Nitrogen balance was not significantly affected by the gel but calcium balance became more markedly negative in the normal subject during the period of gel administration (Table VI). The high calcium intake in the hypoparathyroid patient largely reflects supplements of calcium chloride solution which were required to prevent tetany.

DISCUSSION

Although acute elevations of plasma P rarely alter TmP (7), repeated daily intravenous P infusions result in a reduction in TmP in normal subjects. The decrease in endogenous plasma P together with an increased endogenous P excretion in one subject (D. F.) are suggestive of the findings in hyperparathyroidism. It is of interest that there was no appreciable change in plasma Ca associated with the marked decrease in plasma P. Hence, if parathyroid activity were increased, one must postulate either a separation between the principle altering tubular reabsorption of P and the factor which influences calcium metabolism, or a greater sensitivity of the renal tubule to the parathyroid hormone. The decrease in P reabsorption with P infusions is in keeping with the experiments on rats and humans fed high P diets (8). From our data it is not possible to be certain that the reduction in TmP was a result of parathyroid stimulation. Although there was little change in the TmP of the most severely hypoparathyroid subject, A. W., after P infusions, two mildly hypoparathyroid patients showed reductions of TmP comparable to those seen in the normal individuals. Although we are inclined to the belief that the reduction in TmP in these latter two subjects resulted from stimulation of existing parathyroid remnants, this conclusion cannot be definitely stated from our data.

TABLE V

Effect of aluminum gel on fasting plasma P and on urinary P excretion

Subject	Dietary P *mg./day*	Days on aluminum gel	Plasma P *μM/ml.*	Urine P *μM/min.*
Normals				
C. H.	1,800	None	1.21	9.0
	700	7	1.02	0.3
	3,000	None	1.19	31.0
	600	7	0.91	0.2
D. F.	1,000	None	0.89	7.8
	600	10	0.62	0
J. N.	Ad lib.	None	1.31	8.0
	600	14	1.11	1.0
E. E.	1,800	None	1.02	9.0
	600	11	0.86	0.1
	1,200	None	1.11	14.0
	600	6	0.42	0
R. H.	3,000	None	1.13	39.0
	600	None	1.05	3.0
	600	10	0.80	0
Hypoparathyroid				
A. W.	Ad lib.	None	1.51	2.7
	600	10	0.23	0
	725	None	1.49	3.2
	725	15	0.16	0

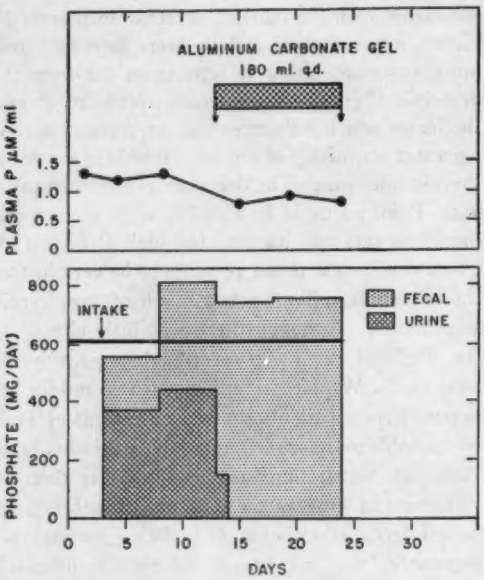

FIG. 1. THE EFFECTS OF ALUMINUM CARBONATE GEL IN A NORMAL SUBJECT UPON PLASMA P AND P BALANCE

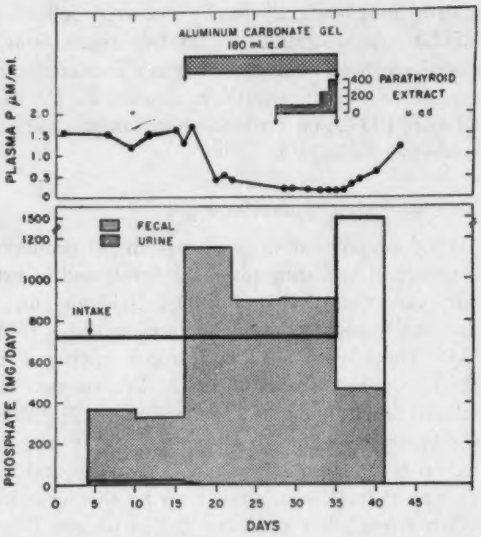

FIG. 2. THE EFFECTS OF ALUMINUM CARBONATE GEL ALONE AND IN COMBINATION WITH PARATHYROID EXTRACT IN A HYPOPARATHYROID SUBJECT UPON PLASMA P AND P BALANCE

The reduction in TmP seen with phosphate infusion does not appear to be the result of the slight fall in serum calcium which occurred in most of these studies. The binding of a portion of the extracellular calcium by intravenously administered di-sodium EDTA resulted in an increase in the urinary excretion of calcium together with a reduction in the oxalate-precipitable plasma calcium. Although the decrease in plasma calcium was comparable to that seen during phosphate infusions, no reduction in TmP was noted after four daily EDTA infusions. Thus, it appears that the reduction in TmP seen with repeated administration of intravenous phosphate solutions results from a direct effect of the phosphate ion rather than from an associated decrease in the ionizable calcium. Furthermore, when calcium gluconate was infused into those patients receiving phosphate, there was no reversal of the phosphate depression of TmP. The infusion of calcium alone has been shown to reduce phosphate excretion as a result of increased tubular phosphate reabsorption (12). This effect is probably due to inhibition of parathyroid secretion (12, 13). Hence, our data indicate that the effect of phosphate administration on tubular phosphate reabsorption is not altered by any parathyroid-inhibiting influence exerted by calcium.

The oral administration of aluminum gels is regularly followed by a reduction in urinary P (10, 14, 15). In normal and hypoparathyroid subjects the administration of the gel resulted in a decrease in plasma P as well as in urinary P, but there was no consistent effect on TmP. Thus, it appears that the decrease in urinary P which follows administration of aluminum gels is explained by a reduction in the filtered load of P delivered to the renal tubules. In the face of an unchanged tubular P reabsorption a fall in filtered P leads to a decrease in P excretion. Conceivably, it is possible to demonstrate in the normal individual a fall in TmP (*e.g.*, with repeated phosphate infusions), but not a rise, because reabsorption of P by the renal tubules under normal conditions approximates their maximal capacity, and hence what approaches a "hypoparathyroid" state. This was demonstrated in a severely hypoparathyroid subject who had determinations of TmP in the untreated state and when he was receiving adequate parathyroid extract to render him "isoparathyroid." The TmP in the second situation was only slightly less than when the patient was un-

TABLE VI

Effect of aluminum carbonate gel on calcium, phosphorus, and nitrogen balance in normal and hypoparathyroid subjects

Subject	Period*	Calcium (gm./day)				Phosphorus (gm./day)				Nitrogen (gm./day)			
		Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance
R. H.	I	0.130	0.102	0.166	−0.138	0.610	0.349	0.207	+0.054	8.56	7.72	1.00	−0.16
Normal	II	0.130	0.072	0.246	−0.188	0.610	0.437	0.372	−0.199	8.56	8.21	1.27	−0.92
	III†	0.130	0.139	0.291	−0.300	0.610	0.030	0.710	−0.130	8.56	6.75	1.10	+0.71
	IV†	0.130	0.226	0.320	−0.416	0.610	0.000	0.757	−0.147	8.56	7.22	1.32	+0.02
A. W.	I	3.406	0.286	2.983	+0.137	0.725	0.142	0.399	+0.184	9.10	8.99	0.76	−0.65
Hypo-	II	3.406	0.213	2.452	+0.741	0.725	0.022	0.349	+0.354	9.10	7.28	0.91	+0.91
para-	IV†	3.406	0.158	3.011	+0.237	0.725	0.003	1.160	−0.438	9.10	6.43	1.64	+1.03
thyroid	V†	3.406	0.241	2.753	+0.412	0.725	0.000	0.901	−0.176	9.10	7.28	1.21	+0.61

* Each period 5 days in length.
† Aluminum carbonate gel 180 ml./day.

treated (6). If this is the case, then a slight rise in TmP might occur following the administration of aluminum gel and yet not be detectable in studies of this type.

Very striking negative phosphorus balances were produced by the administration of aluminum gel to normal and hypoparathyroid subjects. The much larger negative balance seen in the hypoparathyroid subject was accompanied by a much more profound drop in his plasma P. Whether this greater fecal loss of P and greater drop in plasma P can be ascribed to the hypoparathyroidism cannot be stated. It was postulated that the profound fall in plasma P in the hypoparathyroid patient on aluminum gel may have reflected an inability to mobilize skeletal stores of P. The inadequacy of this hypothesis, however, was demonstrated by the absence of change in the plasma P level upon the administration of as much as 400 units of parathyroid extract daily.

The fall in plasma P which usually followed the administration of EDTA is to be compared with the rise observed in patients who are given calcium infusions (13). This decrease in plasma P was clearly not the result of any alteration in P excretion but is consistent with the postulate that the level of plasma Ca influences the distribution of phosphate (16).

CONCLUSIONS

1. Repeated administration of intravenous buffered phosphate solutions to normal males and females resulted in pronounced reduction in TmP.

2. In one severely hypoparathyroid subject phosphate infusions produced a minimal drop in TmP while in two subjects with less marked hypoparathyroidism a reduction in TmP comparable to that seen in normal individuals occurred. Although it seems likely that the effect on tubular reabsorption is exerted through the parathyroids, our data are inadequate to establish this relationship.

3. The reduction in TmP appeared to be correlated with an increase in plasma P rather than from any associated reduction in plasma calcium.

4. The reduction in urinary excretion of phosphate following aluminum gel administration appears to be due primarily to a reduction in endogenous plasma phosphate levels.

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SOME EFFECTS OF INTRAVENOUSLY ADMINISTERED CALCIUM ON INORGANIC PHOSPHATE METABOLISM

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A reduction of urinary phosphate follows the intravenous infusion of calcium in normal individuals (1–3). The fall in phosphate excretion has been ascribed to an inhibition of parathyroid function by the elevated plasma calcium level (2, 3).

A decrease in renal tubular reabsorption of phosphate following the administration of parathyroid extract has been demonstrated (4). Therefore, variations in endogenous parathyroid secretion may be expected to result in changes in phosphate reabsorption by the tubule. If the depression of P [3] excretion following the infusion of calcium is the result of an inhibition of parathyroid activity, it should be accompanied by an increase in P reabsorption by the renal tubule. Such a phenomenon was demonstrated in renal function studies carried out during and immediately following calcium infusions. One might anticipate, further, that alterations in the level of circulating parathyroid hormone would affect the usual response of urinary P to calcium administration. Thus, no fall in urinary P was observed by Howard, Hopkins, and Connor in profoundly hypoparathyroid patients given calcium infusions (3). This observation has been confirmed by our studies. The maintenance of a constant level of circulating parathyroid hormone by the daily administration of a fixed quantity of extract might also be expected to abolish the P response to Ca infusions in normal subjects. This hypothesis, too, was confirmed in the studies to be reported.

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[3] As in the previous papers, the following abbreviations have been used: Ca, calcium; P, inorganic phosphorus; Tm, maximum renal tubular rate of transfer.

METHODS

Most of the subjects and methods employed in this study were described in a previous paper (5). Additional subjects included: R. B., a 22-year-old normal volunteer; T. R., a 70-year-old male with paralysis agitans; E. S., a 7-year-old girl with osteogenesis imperfecta; and V. M., a 40-year-old woman with osteitis deformans and rheumatoid arthritis.

Except where otherwise noted, the studies were carried out with the patients on constant diets containing 130 mg. of Ca and 600 mg. of P. The diets were fed for at least three days before control blood and urine collections were begun.

Urine was collected at the intervals indicated and refrigerated until analyses were performed. The completeness of urine collections was checked on all samples by creatinine determinations, carried out according to the method of Bonsnes and Taussky (6).

Calcium in an amount of 15 mg. per kilogram of body weight was administered intravenously as the gluconate salt, dissolved in 1000 ml. of 0.9 per cent saline over a four-hour period, generally from 8 a.m. to 12 m.

Heparinized venous blood samples for determinations of plasma Ca and P were obtained, one in the fasting state before the infusion was begun, and another at the conclusion of the infusion, before the subject was given lunch, and at least three hours after breakfast. Urine was generally collected in 4, 8, or 12-hour aliquots on one or more days prior to, and on the day following, as well as on the day of the Ca infusion. No ill effects were noted in the patients receiving calcium infusions. The studies of renal function and the administration of parathyroid extract were carried out as described in earlier papers (4, 5).

RESULTS

Effects of Ca infusions on P excretion in normal subjects

In 33 studies in 9 subjects with normal parathyroid function, a 4-hour infusion containing 15 mg. of Ca per kilogram of body weight led to a fall in the 24-hour urinary P excretion of 9 to 71 per cent. The decrease in urinary P was most pronounced during the 8 to 24 hours following the start of the Ca infusion. In most subjects a *rise*

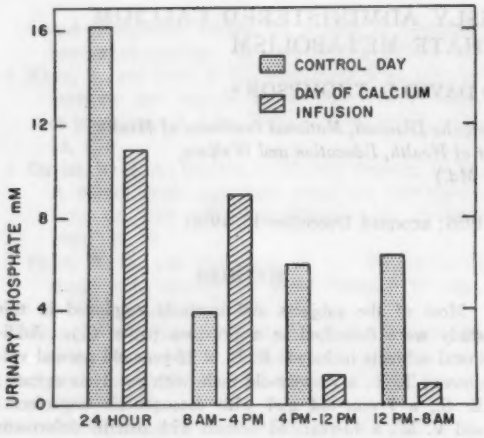

FIG. 1. THE EFFECTS OF INTRAVENOUS CALCIUM ON THE URINARY EXCRETION OF PHOSPHATE IN R. H., A NORMAL SUBJECT

The calcium was infused from 8 a.m. to 12 m.

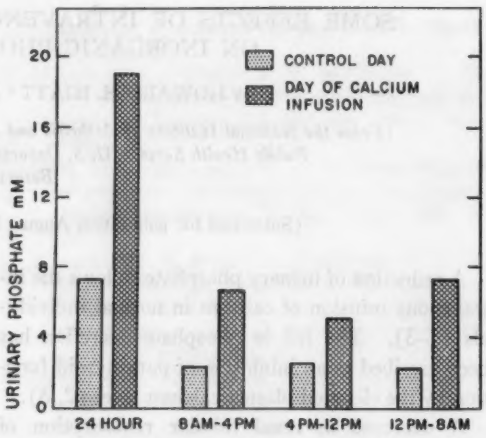

FIG. 2. THE EFFECTS OF INTRAVENOUS CALCIUM ON THE URINARY EXCRETION OF PHOSPHATE IN A. W., A PATIENT WITH PROFOUND HYPOPARATHYROIDISM

The calcium was infused from 8 a.m. to 12 m.

in P excretion was observed during the 4 hours in which Ca was infused, and in several subjects this increase continued during the four hours following the completion of the Ca infusion (Figure 1). Hence the inhibitory effect of the calcium infusion on urinary P was actually greater than was apparent from a measurement of only the total 24-hour P excretion.

Effects of Ca infusions on P excretion in patients with parathyroid disease

In the only subject with profound hypoparathyroidism studied the infusion of calcium on four occasions led to a marked rise in P excretion, not only during the Ca infusion, as in normal subjects, but throughout the remainder of the 24-hour period (Figure 2). One subject who had mild, but unequivocal clinical and laboratory manifestations of hypoparathyroidism had a decrease in urinary P excretion following a calcium infusion (Figure 3). In this subject no significant change in P excretion was observed upon examination of only the data for the entire 24-hour period. The fall in urinary P during the period from 11 p.m. to 8 a.m., however, was unequivocal, and far greater than spontaneous variations observed in this patient's P excretion on several successive days during which no calcium infusions were given. A patient with hyperparathyroidism, later found to have a parathyroid adenoma, had no change in urinary P following a calcium infusion.

Effect of dietary intake on the response of P excretion to Ca infusions

All of the studies reported above and many of those described in the literature were carried out with the subjects on constant diets containing small quantities of Ca and P. Changes in the intake of these minerals were shown to have no qualitative influence on the decrease in urinary P which followed Ca infusions (Table I). In most subjects, however, the fall in urinary P relative to the excretion on the control day was more striking on the low Ca-low P diet than when the intake of Ca or P was increased. Variations in urinary P when the intake of this mineral was inconstant were so great that no conclusions could be drawn concerning the influence of Ca infusions under such conditions. Of interest is the fact that an increase in dietary Ca while the intake of P was held constant led to a sustained fall in urinary P comparable to that seen following the intravenous administration of Ca (Table I, C. H., R. H.). Complete metabolic balance studies indicated that the increase in Ca intake resulted in an increased P storage, rather than a shift of P from urine to the stools.

TABLE I

Effects of calcium infusions on plasma and urinary phosphate at different dietary levels of calcium and phosphorus

Subject	Date	Intake		Plasma P		Urine P	
		Calcium mg.	Phosphorus mg.	Before μM/ml.	After μM/ml.	Control mM/24 hr.	Exp'tal mM/24 hr.
C. H.	6/2/54	130	600	1.25	1.57	24.1	10.0
	9/11/54	130	600			21.2	6.1
	12/18/54	130	600	1.16	1.60	17.4	8.3
	2/8/55	1,200	600	1.21	1.54	10.0	5.1
R. H.	10/13/54	130	600	1.27	1.53	15.2	8.4
	2/5/55	1,500	600	1.00	1.11	3.7	1.5
	3/9/55	130	1,600	1.09	1.55	41	29
W. R.	9/21/54	130	600	1.16	1.65	20	14
	11/15/54	200	1,500	0.86	1.34	40	33
V. M.	12/6/54	130	600	1.08	1.31	9.8	5.7
	12/10/54	130	600	1.00	0.87	10.9	7.7
	12/17/54	130	1,800	1.19	1.14	30.8	24.4
D. F.		610	1,015	1.05	1.58	27	24

Effect of administration of parathyroid extract on the response of P excretion to Ca infusions

The fall in urinary P during calcium infusions in normal subjects and the absence of such a phenomenon in severely hypoparathyroid individuals have led to the postulate that the decrease in P excretion results from a diminution of parathyroid secretion. To test this hypothesis parathyroid extract was administered at several dosage levels to normal subjects. After the completion of one or more control studies, parathyroid extract was given at each dosage level until the 24-hour urinary excretion of P was reasonably constant and continued through the day on which the calcium was infused. It was reasoned that if the decrease in urinary P were the result of an inhibition of parathyroid secretion, a Ca infusion should have no effect on P excretion in a subject receiving a constant amount of exogenous parathyroid extract. Such was found to be the case, the effect of Ca infusions on P excretion becoming progressively less with increasing dosage of parathyroid extract, and finally disappearing when the patient was receiving 300 units of parathyroid extract daily (Figure 4). (Why the urinary P excretion in the study described in Figure 4 was lower with the subject receiving 300 units of parathyroid extract daily than with 200 units is not known. The same lot of parathyroid was used, and the serum Ca at the 300-unit level was 11.45 mg. per cent, as compared with 10.00 at 200 units.) A similar phenomenon was observed in 4 other subjects (Table II). The daily administration of 200 units of parathyroid extract to the subject with severe hypoparathyroidism led to a restoration to normal of the plasma and urinary Ca and P, but had no effect on the profound increase in P excretion observed in this subject in response to Ca infusions.

Effect of Ca infusions on plasma P

The intravenous infusion of Ca led to an increase of up to 50 per cent in the plasma P in al-

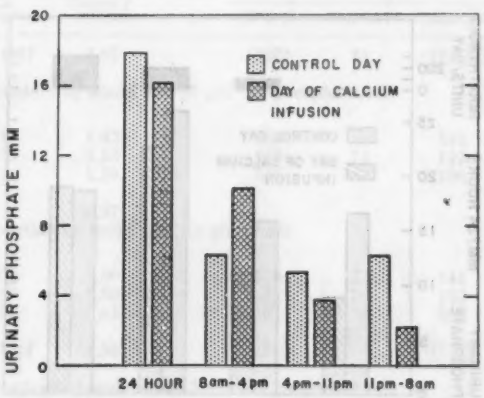

FIG. 3. THE EFFECTS OF INTRAVENOUS CALCIUM ON THE URINARY EXCRETION OF PHOSPHATE IN L. D., A PATIENT WITH MILD HYPOPARATHYROIDISM

The calcium was infused from 8 a.m. to 12 m.

TABLE II

Effects of calcium infusions on plasma and urinary phosphate under influence of parathyroid extract

Subject	Date	Parathyroid extract		Plasma Ca	Plasma P		Urine P	
		units/day	*days*	*mg. per cent*	Before *μM/ml.*	After *μM/ml.*	Control *mM/24 hr.*	Exp'tal *mM/24 hr*
C. H.	12/18/54	0		9.81	1.16	1.60	17	8
	11/12/54	200	6	9.00	1.33	1.47	18	17
	7/2/54	2,000	1	12.23	1.11	1.48	44	62
W. R.	9/21/54	0		10.35	1.16	1.65	20	14
	5/9/55	1,500	3	13.87	0.95	0.51	58	64
V. M.	12/6/54	0		9.50	1.00	0.87	10.9	7.7
	12/13/54	200	4	10.25	0.99	0.99	12.8	12.0
E. P.	5/17/55	0		10.30	1.24	1.43	19.9	11.9
	3/24/55	600	4	13.15	0.95	1.14	34	34

most all of the subjects studied (Tables I and II). The plasma P reached its maximum level at the completion of the Ca infusion and was usually still elevated 8 to 16 hours later, even when the plasma Ca had returned to its control level. The increase in plasma P bore no constant relationship to changes in P excretion. It was as great in the hypoparathyroid subject whose urinary P increased following the Ca infusion as it was in most of the normal subjects. No increase in plasma P was seen on several occasions in V. M., a patient with osteitis deformans, and in some, but not all of the normal subjects rendered hyperparathyroid by the administration of parathyroid extract (Table II).

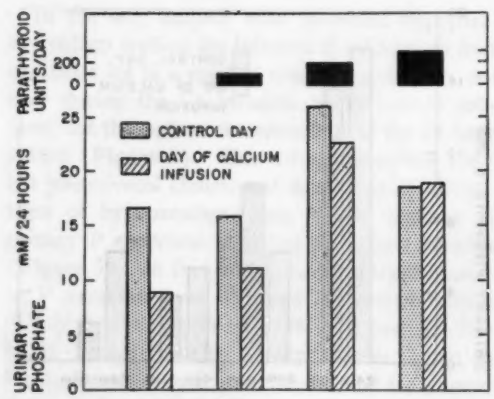

FIG. 4. THE INFLUENCE OF SEVERAL DOSAGE LEVELS OF PARATHYROID EXTRACT ON THE RESPONSE OF THE URINARY P TO CA INFUSIONS IN R. H., A NORMAL SUBJECT

Effects of Ca infusions on renal function

To investigate further the mechanism of the changes in phosphate excretion resulting from Ca infusions, renal clearance studies were carried out before, during, and after such infusions in normal subjects. Phosphate excretion in one subject was found to be 20 μM per minute prior to, 25 μM per minute during, and 7 μM per minute 16 hours following a calcium infusion (Table III). The rise in P excretion during the Ca infusion was found to be the result of an increase in filtered phosphate, which, in turn, was consequent to a rise in plasma phosphate. The reabsorbed phosphate was shown to be *increased* during the Ca infusion. Sixteen hours following the infusion the filtered phosphate was still considerably higher than the pre-infusion level, again as a result of an increased plasma phosphate (which had not yet returned to its control value). At this time since the tubular reabsorption of P remained high, there was a fall in the excretion of P.

Because the Ca infusions led to a rise in plasma P, the aforementioned studies did not permit an assessment of renal tubular reabsorption under conditions of equal filtered loads of phosphate. Hence, similar studies were carried out, but with the intravenous administration of enough phosphate *before* and *following* the Ca infusion to raise the serum phosphate to the level anticipated *during* the Ca infusion (Table IV, Figure 5). In the study of R. H. (Table IV) it is clear that at equivalent loads of filtered P approximately 40 per cent more P was reabsorbed during, and 16

TABLE III

Patterns of renal excretion of phosphate before (A), during (B), and after (C) infusion of calcium *

	Time	Urine flow ml./min.	C_{PAH} ml./min.	Plasma Ca mg./100 ml.	C_{In} ml./min.	Plasma P μM/ml.	Phosphate (μM/min.) Filtered	Excreted	Reabsorbed
5/11/55	8:53–9:18			9.10		1.02		7.4	
a.m.	9:48	Priming dose of inulin and PAH							
	9:52	Sustaining infusion of inulin and PAH begun at 3.8 ml./min.							
A	10:35–11:14	14.1	598	9.20	125	0.97	121	18	103
	11:14–11:38	13.7	638		125	0.95	119	19	100
	11:38–12:04	13.1	625	8.90	124	0.96	119	22	97
5/11/55									
p.m.	5:30	Sustaining infusion of inulin, PAH, and Ca gluconate begun at 3.8 ml./min.							
	7:47–8:08	14.3	545	13.20	119	1.41	168	23	145
B	8:08–8:25	14.0	618		123	1.44	177	24	152
	8:25–8:43	15.5	621	14.00	126	1.44	181	27	154
	9:30	Infusion of calcium completed							
5/12/55	8:50–9:10	9.7		9.20		1.35		0.3	
a.m.	9:25	Priming dose of inulin and PAH							
	9:28	Sustaining infusion of inulin and PAH							
C	10:19–10:51	11.4	625	9.74	117	1.34	157	5	152
	10:51–11:17	11.2	647		118	1.33	157	7	150
	11:17–11:43	11.4	620	9.43	119	1.41	168	9	159

* R. H., normal female.

hours following a Ca infusion than during the control period. The excretion of phosphate during the Ca infusion was increased over the control (26 μM per minute *vs.* 12). However, at comparable filtered P loads, the P excretion is *less* during the Ca infusion than during the control periods (26 μM per minute as compared with 76). Indeed, a rise in P reabsorption occurred during

TABLE IV

Patterns of renal excretion of phosphate before (A), during (B), and after (C) infusion of calcium *

	Time	Urine flow ml./min.	Plasma Ca mg./100 ml.	C_{In} ml./min.	Plasma P μM/ml.	Phosphate (μM/min.) Filtered	Excreted	Reabsorbed
6/23	9:00–9:30	11.1	9.22	(118)†	1.02	(120)	12	(108)
a.m.	9:55	Priming dose of inulin						
A	10:00	Begin sustaining infusion containing inulin and 30 μM Na phosphate/ml. at 3.8 ml./min.						
	11:00–11:35	10.6		119	1.42	168	54	114
	11:35–12:13	9.4		121	1.53	185	73	112
	12:13–12:35	10.5		116	1.59	185	79	106
6/23	5:25		9.35		0.97			
p.m.	5:30	Begin sustaining infusion containing inulin and Ca gluconate at 3.8 ml./min.						
B	8:00–8:20	14.1	11.05	109	1.50	164	23	141
	8:20–8:42	14.6		119	1.50	179	26	153
	8:42–9:30	14.8	11.85	118	1.65	191	28	163
6/24	9:00–9:30	9.41	9.21	(117)†	1.36	(159)	4	(155)
a.m.	9:44	Priming dose of inulin						
C	9:47	Begin sustaining infusion containing inulin and 15 μM Na phosphate/ml. at 3.8 ml./min.						
	11:20–11:46	9.7		118	1.60	189	39	150
	11:46–12:09	10.3	8.76	115	1.62	186	44	142

* R. H., normal female.
† Not measured; average of succeeding periods.

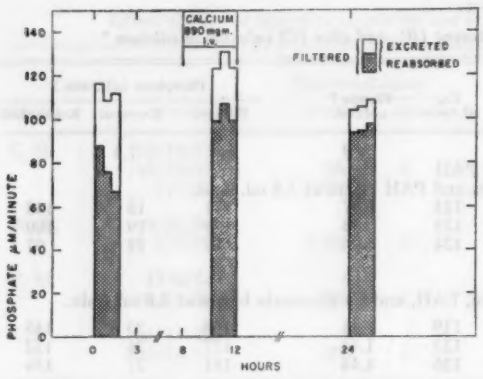

FIG. 5. FILTERED, REABSORBED, AND EXCRETED PHOSPHATE BEFORE, DURING, AND FOLLOWING THE INFUSION OF CA TO E. P., A NORMAL FEMALE

the Ca infusion. Sixteen hours later the plasma P, although not yet back to its control level, 1.02 μM per ml., had fallen from its peak of 1.65 μM per ml. to 1.36 μM per ml. The consequent decrease in filtered load along with the still-elevated reabsorbed phosphate resulted in a marked reduction in the excretion of P.

Calcium retention

The portion of the intravenously administered calcium retained was considered to be the difference between the amount of calcium administered and the increase in urinary calcium on the day of the calcium infusion over the excretion on the previous day. Since fecal calcium was not measured during these studies, the figure for calcium retention is only approximate; previous work, however, has indicated that only a small proportion of intravenously administered calcium appears in the stool (7). The calcium retention approximated 70 per cent of the administered dose in 4 of 5 subjects without parathyroid disease (Table V). In all subjects the fraction of calcium retained was remarkably constant when determined on more than one occasion, even several months apart. The low urinary excretion and high retention of Ca by A. W. was undoubtedly ascribable to the low baseline level of plasma Ca. In this individual, the Ca infusion led to a rise in plasma Ca from approximately 7.0 mg. per cent to 10 mg. per cent. Hence, the filtered load of calcium was undoubtedly lower than that occurring in normal individuals.

DISCUSSION

The decrease in urinary P which follows the intravenous administration of Ca appears to be mediated by the parathyroids. We have confirmed the reports of others (3, 8) that markedly hypoparathyroid subjects respond to Ca infusions with an increase in urinary P. In addition, we have observed the abolition of the usual response to Ca infusions in normal subjects by the administration of parathyroid extract. The amount of extract required to achieve this end was surprisingly large; in one subject P excretion was inhibited by Ca infusions until a daily dosage of 300 units was reached. We had postulated that the response to Ca would disappear upon the administration of an amount of extract approximating the endogenous parathyroid secretion. On the basis of maintenance requirements of patients with hypoparathyroidism, we had estimated this figure to be considerably less than 300 units daily.

Our data support the advice that studies of the influence of Ca on P excretion be done only after at least three days on a diet constant in calcium and phosphorus content, and preferably low in these minerals. All of our subjects, normal, hypoparathyroid, and those with induced hyperparathyroidism, showed a rise in urinary P during the period of Ca infusion. Hence, if this procedure be employed as a measure of parathyroid function, its usefulness would be increased by collecting urine during the period 8 to 24 hours following the start of a four-hour Ca infusion, and during a corresponding period on the control day. Since our subject with mild hypoparathyroidism showed a "normal" response to Ca administration, it is unlikely that this test can be considered a sensitive index of parathyroid insufficiency.

Whether the level of serum Ca governs parathyroid secretion remains unknown. No detectable change in serum level occurred in patients whose dietary Ca was increased, but a profound and sustained fall in P excretion followed. The apparent relationship of serum or dietary Ca to parathyroid secretion does not, of course, preclude the possibility that other factors influence both parathyroid function and phosphate transport by the renal

TABLE V

Effect of calcium infusions on urinary calcium

Subject	Date	Calcium infused mg.	Urinary calcium mg./24 hours		Administered Ca "retained" %
			Control	Day of Ca infusion	
C. H. (Normal)	6/54	975	121	411	70
	9/54	982	89	335	75
	11/54	982	90	403	68
W. R. (Normal)	11/54	982	125	481	64
R. H. (Normal)	10/54	980	63	610	44
	10/54	980	104	670	43
	1/55	890	94	547	49
	1/55	890	125	580	49
D. F. (Normal)	1/55	1,250	38	358	74
V. M. (Osteitis deformans)	12/55	515	90	229	75
	12/55	515	111	185	66
	12/55	515	73	204	74
	12/55*	515	168	324	70
A. W. (Hypoparathyroid)	9/54	960	59	181	87
	1/55	890	20	72	91
	2/55	890	62	96	95

* On 200 units of parathyroid extract daily.

tubule. Indeed, a profound depression of TmP was demonstrated following the intravenous administration of large quantities of P, and this phenomenon was not reversed by the repeated intravenous administration of Ca (9).

The mechanism of the increase in plasma P which follows a rise in serum Ca has not been demonstrated in these studies. It is clear, however, that it is not dependent on the integrity of the parathyroids, and that it is independent of changes in the urinary excretion of P. Evidence that the elevation of serum Ca leads to a shift of P from the cells has been advanced by Chen and Neuman (10).

Our studies demonstrate that the rise in urinary P seen *during* a Ca infusion reflects an increase in filtered P, in turn the result of the rise in plasma P. Of interest is the observation that the reabsorbed P reached its maximal level within three hours after the start of the Ca infusion. If the change in tubular transport of P reflects an inhibition of parathyroid secretion, and if the duration of action of the parathyroid hormone already circulating prior to the Ca infusion approximates the six hours found to be the case in the dog by Hastings and Huggins (11), then one might have expected phosphate reabsorption to have been unchanged for several hours. There exists the possibility that during the period of the elevated serum Ca level the rise in reabsorbed P as measured appears to be higher than it actually is, as a result of the presence of a non-filtrable Ca-P complex. This seems unlikely, however, in light of the ultra-filtrability studies of Hopkins, Howard, and Eisenberg (12), and also because of the close correspondence of the figures for reabsorbed P during the Ca infusions and several hours later when the plasma Ca had returned to normal levels. The increase in tubular reabsorption of P during the Ca infusion recalls a similar phenomenon observed by Foulks following the administration of sulfate to dogs (13). In both instances the rise in tubular reabsorption was accompanied by an elevation of serum P, and, hence, presumably by a decrease in intracellular P. Foulks postulated that a lowered intracellular P will effect a rise in the quantity of P reabsorbed by the renal tubules (13).

Schilling and Laszlo (14) have suggested that the avidity of the skeleton for calcium can be estimated from the proportion of an intravenously administered dose of Ca not appearing in the urine.

They have found that normal subjects retain approximately 70 per cent of the Ca infused, while cancer patients with osteolytic processes retain less, and those with osteoblastic metastases retain more. The behaviour of most of our normal subjects corresponded closely to that reported by Schilling and Laszlo, and the response of those subjects tested on more than one occasion showed little variation. The consistently high excretion of the administered Ca by one young normal control subject without evidence of parathyroid or bone disease, however, suggests that this test cannot by itself be used as an index of the presence of bone disease.

SUMMARY

1. The intravenous administration of calcium gluconate to normal subjects results in a diminution in the urinary excretion of phosphorus. This phenomenon occurs as a result of increased renal tubular reabsorption of phosphate.

2. This effect of administered calcium appears to require normally functioning parathyroid glands, since it is not seen in patients with profound hypoparathyroidism or hyperparathyroidism, and it can be abolished in normal subjects by the prior administration of parathyroid extract.

3. No change, or even an elevation in P excretion occurs *during* the administration of calcium. Hence, more striking reductions in P excretion are detected if urine is collected during the 8 to 24 hours after the four-hour infusion of calcium is begun, and compared with the corresponding period on the control day.

4. The response of urinary P excretion to calcium administration is not a sensitive test for hypoparathyroidism, since patients with clinically apparent, but mild hypoparathyroidism may show a pattern similar to that of normal subjects.

5. The percentage of administered calcium retained by a given individual is quite constant on repeated testing. However, unexplained variability occurs in the amount retained by different subjects.

6. The intravenous infusion of calcium leads to a prompt rise in plasma phosphate in almost all subjects. The rise occurs before there is any reduction in urinary excretion of P, and hence must represent mobilization from intracellular stores. In some individuals large doses of parathyroid extract abolish this elevation of plasma P.

ACKNOWLEDGMENTS

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THE DYNAMICS OF SWALLOWING. I. NORMAL PHARYNGEAL MECHANISMS

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Swallowing is a complex mechanism in which the functions of the central and autonomic nervous systems are integrated to produce a smooth and speedy transference of the bolus from the mouth to the stomach. The pharyngeal phase of deglutition is the most rapid, being usually of less than a second's duration, and for this reason has been difficult to analyze fully.

The forces involved in the propulsion of the bolus through the pharynx have been a source of controversy. Some have maintained that the bolus is advanced under positive pressure by the action of the muscles of the tongue and pharynx (1, 2); yet others have postulated negative intrapharyngeal pressures (3), in particular Barclay (4), who believed that, after preliminary elevation, the pharynx suddenly descended thereby sucking the bolus downward. Barclay's concepts have few adherents today, but transient negative pressures, uncertain as to origin and purpose, do appear during swallowing. Recently, for example, Fyke and Code (5) have recorded positive pressures from the upper pharynx and negative ones from the cricoid level on swallowing.

It is generally assumed that the pharynx in the resting state is closed off from the esophagus, and although the existence of an anatomical sphincter in this region is controversial, the lower part of the inferior constrictor and perhaps the adjoining circular muscle of the esophagus are believed to perform this function (6, 7).[1] A cricopharyngeal sphincter has been demonstrated radiographically by Templeton and Kredel (8) and its functioning has been illustrated by cinefluorography by Ramsey, Watson, Gramiak, and Weinberg (9). Whether the cricopharyngeus represents a true sphincter with powers of active relaxation and contraction, or whether it functions in a passive manner, as some have assumed (8), is debatable. Radiographic techniques have been of limited value in deciding this question, as the area has been studied principally by observing the passage of barium, whereas many of the dynamics of swallowing precede the bolus. Also, radiography yields only indirect information about the forces concerned in the movement of the bolus.

To investigate the mechanism of swallowing and the nature and magnitude of the forces involved, we have recorded intraluminal pressures simultaneously from three levels in the pharynx and esophagus. At the same time rapid serial radiographs have been taken to enable interpretation of pressure records in terms of anatomical function and movement of the bolus. These observations form the basis of this report.

METHODS

Intrapharyngeal pressures were recorded through water-filled, open-ended polyethylene tubes attached to Sanborn electromanometers and a four-channel, direct-writing Sanborn recorder. For measuring resting pressures in the pharynx and upper esophagus, a single tube was used. This tube had an inside diameter of 1.7 mm. and was equipped with a metal tip to enable radiographic visualization. A small strip of lead was attached to the skin over the cricoid cartilage to act as a marker in the radiographic localization of the position of the tube. With the subject supine, the tube was inserted into the mid-esophagus to the 30 cm. mark. Then, under fluoroscopic control, it was withdrawn centimeter by centimeter, and intraluminal pressures were recorded in the esophagus and pharynx at each level.

For measuring swallowing complexes, three such tubes were fastened together so that the distance between the distal and middle tips was 2 cm., and that between the middle and proximal tips 4 cm. These tubes were inserted into the pharynx and esophagus through the nose.

Polyethylene tubes rather than nylon cardiac catheters were used because their flexibility permitted them to

[1] The terms "cricopharyngeus" and "cricopharyngeal sphincter" are hereafter used to include the entire sphincteric mechanism.

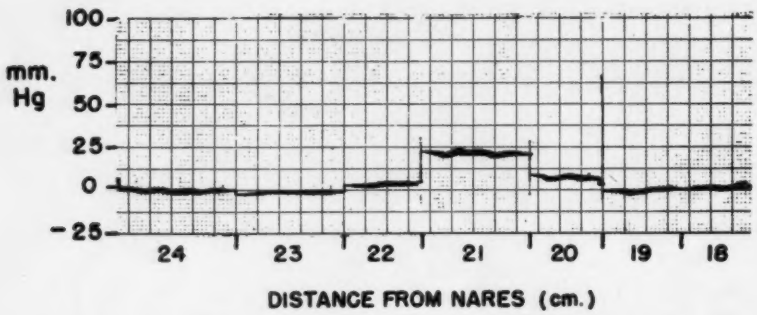

FIG. 1. RESTING INTRALUMINAL PRESSURES IN THE ESOPHAGUS AND PHARYNX OBTAINED BY INSERTING THE RECORDING TIP INTO THE UPPER ESOPHAGUS AND WITHDRAWING CENTIMETER BY CENTIMETER

The zone of high resting pressure at the cricoid level is demonstrated.

move up and down with the pharynx during swallowing. Hence, their tips remained in a relatively constant position with respect to the pharyngeal and esophageal wall. In addition, their flexibility greatly reduced discomfort from pharyngeal irritation. In preliminary experiments simultaneous recordings were made with a polyethylene tube and a nylon catheter fastened together with their tips at the same level. The records obtained were identical, and therefore the compressibility of the polyethylene tubing was not such as to alter the record.

Studies were made upon fasting subjects in both the sitting and supine positions. When the subject was supine, the posterior border of the cricoid cartilage was taken as the point of zero pressure. With the subject sitting, the zero points were adjusted to the heights of the recording tips. In this way absolute pressures were determined with sufficient accuracy to permit comparison of pressures at the three recording sites. Swallows of 10 to 20 ml. of water, or barium suspension, or small quantities of chewed or unchewed bread were used.

Serial radiographs were taken in the anteroposterior and lateral planes at speeds of 6 to 12 per second by means of an Elema machine using 12-inch roll film. The following radiographic factors were employed: 300 ma, 110 Kv, 1/60 sec. A lead, taken from the primary winding of the transformer for the lateral X-ray tube and passed through a transformer resistance box, was fed into the fourth channel of the recorder. In this way, the exact times of exposure of the films in relationship to intraluminal pressure changes were recorded.

Pressure records from the pharynx and upper esophagus were obtained in 30 healthy male subjects; in 22, pressures alone were measured, and in 8, simultaneous pressure measurements and radiographic studies were made.

TABLE I

Resting intraluminal pressures (mm. Hg) at the junction of pharynx and esophagus

Subject	Esophagus		At cricoid	Pharynx	
	2 cm. below cricoid	1 cm. below cricoid		1 cm. above cricoid	2 cm. above cricoid
O. P.	−10	10	8	2	0
A. R.	− 4	60	20	5	2
C. T.	2	22	30	7	2
V. G.	− 8	−5	20	25	8
G. S.	− 7	7	25	40	−3
M. A.	− 2	0	40	8	0
R. S.	− 7	10	28	0	0
A. H.	− 3	12	30	2	2
R. M.	0	17	32	20	3
J. C.	− 3	−3	27	12	0
S. A.	0	25	25	0	−2
T. H.	0	10	28	16	3
T. R.	−10	18	55	8	0
T. B.	− 2	0	30	23	5
J. B.	3	10	60	21	2
A. B.	− 4	5	45	30	5

RESULTS

Resting pressures in the pharynx and upper esophagus

Measurements were made in 16 subjects (Table I). In the upper part of the esophagus, resting pressure was usually a few millimeters below atmospheric, and respiratory fluctuations were slight and often absent. When the recording tip was withdrawn to the region of the cricoid cartilage, elevation of the resting intraluminal pressure was seen (Figure 1). At this point, further withdrawal of the tip was opposed by a sense of resistance; once through this zone, the tip could be returned to the esophagus only when the subject swallowed. The extent of this zone of elevated pressure varied considerably from subject to subject; in some it was only 1 cm. in length, and in these it was difficult to maintain the recording tip in the high pressure zone. Others showed an elevated resting pressure over a distance of 4 cm. with a step-like increase and decrease on either side of the point of maximal pressure, which usually corresponded with the level of the cricoid cartilage. The maximal elevation of resting pressure varied between 10 and 60 mm. Hg, with a mean of 35 mm. Hg.

The height of the elevation of pressure in the cricoid area varied considerably from subject to subject, and repeat measurements in the same subject did not always give the same results. This variability may be explained in part by the fact that the recording tip may not always have been placed in exactly identical positions. It seemed more likely, however, that resting pressures actually did change during the course of some studies; in fact, an increase or decrease in resistance to the passage of the tube could sometimes be appreciated by the intubator.

Radiographs confirmed that the zone of high resting pressure corresponded with the region of the cricopharyngeal sphincter. As shown in Figure 5, plate 4L, the distal tip, which was in the high pressure zone, may be seen just below the air-filled pharynx and at the level of the cricoid. The sphincter, outlined by a swallow of barium (Figure 4, plate 13A), corresponded with the position of the distal tip.

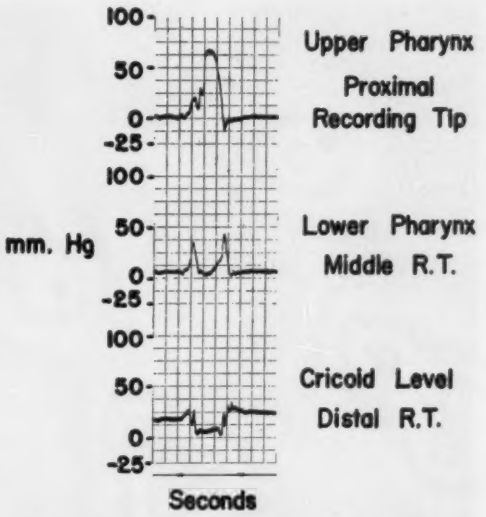

FIG. 2. SIMULTANEOUS SWALLOWING PRESSURES FROM THE UPPER AND LOWER PHARYNX AND THE LEVEL OF THE CRICOPHARYNGEUS

Pressure changes in pharynx and upper esophagus on swallowing

For descriptive purposes, four types of swallowing pressure complexes will be outlined: esophageal, cricopharyngeal, lower and upper pharyngeal.

Esophagus. Deglutition pressure changes in

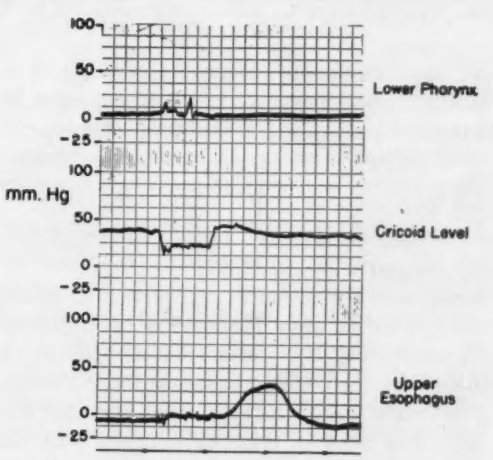

FIG. 3. SIMULTANEOUS SWALLOWING PRESSURES FROM THE LOWER PHARYNX, CRICOPHARYNGEAL LEVEL, AND UPPER ESOPHAGUS

Time intervals in seconds.

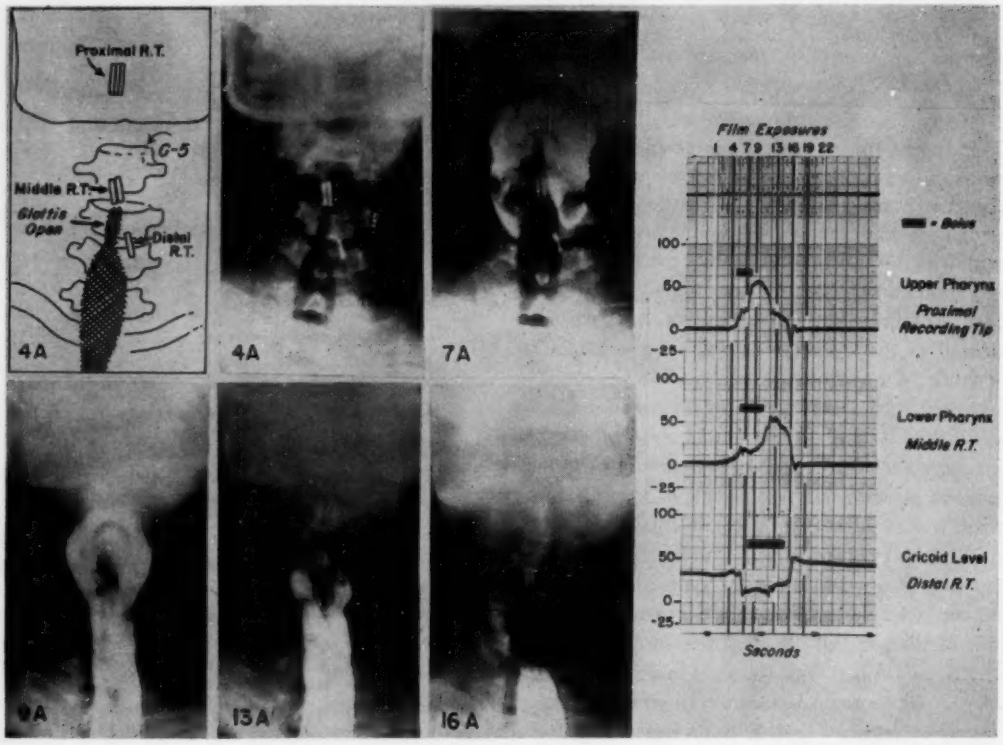

FIG. 4. RADIOGRAPHS TAKEN DURING THE RECORDING OF INTRAPHARYNGEAL PRESSURE CHANGES ON SWALLOWING BARIUM SUSPENSION

The corresponding pressure record is shown on the right, the vertical lines indicating the times of film exposure. The heavy horizontal bar indicates the passage of the bolus in relation to the tracing.

the upper esophagus (Figure 3) consisted of an initial transient negative deflection succeeded by a positive plateau and a final positive wave, the whole complex lasting about 4 to 5 seconds. These findings conform with previous descriptions (10, 11).

Cricopharyngeal level. In the zone of high resting pressure a completely different type of swallowing complex occurred. A rectangular downward deflection now became the basic pattern. On swallowing, the pressure fell abruptly to, or just above, the resting esophageal level, although occasionally a small positive wave preceded this fall. This drop in pressure coincided with the beginning of the positive plateau in esophageal records (Figure 3). After the downward dip, cricopharyngeal pressure either remained stationary or rose slightly for an interval of 0.5 to 1.2 seconds; thereafter, pressure rose, as abruptly as it had fallen, to a resting level, often higher (Figure 2) and sometimes lower than the initial level. This apparent change in resting pressure may have been caused by displacement of the recording tip during swallowing, but actual change in sphincteric tone may have occurred.

On occasions, a final slow positive wave, comparable in amplitude and duration with the final peristaltic pressure wave in the esophagus, was recorded after the rectangular downward complex. Simultaneous radiography, however, suggested that this was due to displacement of the recording tip downward into the upper esophagus.

Lower pharynx. Immediately above the zone of high resting pressure, double-peaked, purely positive swallowing complexes occurred. The relative height of the two peaks varied, but in gen-

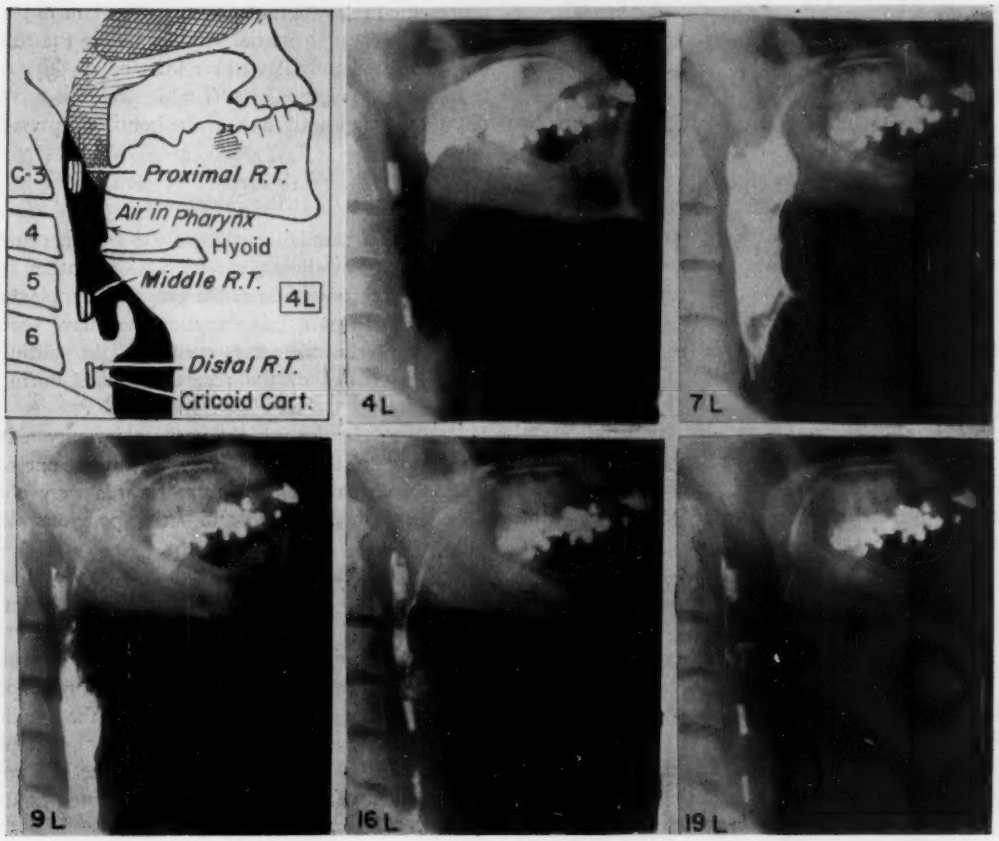

FIG. 5. LATERAL FILMS EXPOSED SIMULTANEOUSLY WITH THE ANTEROPOSTERIOR FILMS SHOWN IN FIGURE 4
R.T. = recording tip.

eral the second was the larger, ranging between 15 and 80 mm. Hg in amplitude. The time interval between these peaks was of the order of 0.4 to 1 second and differed from one swallow to another in the same subject. The second peak occurred immediately before the abrupt return to high resting pressure at the cricoid level (Figure 2).

Upper pharynx. There was no sharp demarcation between the pressure patterns seen in the upper and lower parts of the pharynx. The first pressure peaks corresponded in time and amplitude throughout the pharynx, but the higher the recording tip the closer did the second peak follow behind the first, until in the upper pharynx the two almost merged. Here the amplitude of the second peak usually was greater than the first.

Description of the swallowing act on the basis of correlated radiologic findings and pressure measurements

Upward pressure of the tongue, from the tip backwards, against the hard palate initiated swallowing by thrusting the bolus backward into the pharynx. At the same time, the nasopharynx was closed off by the soft palate, the larynx was elevated, and the vocal cords approximated. The initial negative pressure deflection in the upper esophagus corresponded with the elevation of the larynx, and hence might have represented stretching of the esophagus (12). However, simultaneous intragastric pressure records indicated that a sudden increase in intragastric pressure occurred at this instant, and it is, therefore, also possible

that the negative deflection in the esophageal record was due to the sudden inspiration (Schluckathembewegung) which has been recorded early in swallowing (3).

The movement backward of the bolus into the pharynx, which was now a closed cavity, corresponded with the first peak of the intrapharyngeal pressure record. However, immediately after the head of the bolus entered the pharynx, the cricopharyngeus appeared to open. This occurred synchronously with the abrupt fall in pressure at the cricoid level (Figure 5, plate 7L) and the commencement of the positive plateau in the upper esophagus (Figure 3). The bolus then passed down the pyriform sinuses into the esophagus (Figure 4, plates 7A and 9A) as intrapharyngeal pressure dropped to form the trough between the two peaks. The head of the bolus moved rapidly down the pharynx at a speed of 40 to 50 cm. per second but slowed down at the cricoid region and took longer to pass. This slowing probably took place because the esophageal lumen is narrower than that of the pharynx. It did not appear due to delay in relaxation of the cricopharyngeus as this occurred before the head of the bolus reached the cricoid level (Figure 4, plate 7A).

Successive contraction of the superior, middle and inferior pharyngeal constrictors then took place, rapidly forcing the remainder of the bolus into the esophagus (Figure 4, plate 13A; Figure 5, plates 9L and 16L). The wave of constrictor activity coincided with the second major peak of intrapharyngeal pressure. Radiography indicated that this wave of constriction passed down the pharynx in approximately 1 second. This corresponded with a rate of 5 to 10 cm. per sec., calculated for the wave of constriction on the basis of a time interval of 0.4 to 0.8 second between the second peaks of the upper and lower pharyngeal records.

The second pharyngeal pressure peak was followed immediately by closure of the cricopharyngeal sphincter, coinciding with the abrupt return to high resting pressure at the cricoid level. The larynx descended, the pharynx filled with air and respiration was resumed. The final brief negative wave in the upper pharyngeal record occurred immediately before the pharynx refilled with air (Figure 5, plates 16L and 19L). It may have represented relaxation of the muscular walls of the pharynx creating a partial vacuum before the soft palate relaxed and allowed air to re-enter.

In the esophagus, the peristaltic pressure wave did not develop until most of the bolus had passed downward.

Variation in swallowing complexes

In spite of standardization of the amounts and frequency of swallows, considerable variation in duration and amplitude of the pressure complexes was seen. In part, this variation may have been caused by technical factors such as slight shifting of the apertures of the recording tubes during swallowing.

Posture. In the upright position, gravity aids the passage of the bolus. However, in not one of five subjects in whom pressure records were obtained both in the supine and sitting positions did posture cause appreciable change.

Type of bolus. In three subjects swallows of water were followed by swallows of chewed or unchewed bread. Although no appreciable difference in swallowing complex occurred with chewed bread as compared with water, swallowing lumps of bread usually caused an increase in the amplitude and duration of pressure complexes, perhaps representing powerful constrictor contractions required to force the bulky and unlubricated bolus through the pharynx.

Drinking without swallowing. Certain individuals have the faculty of pouring fluids down into the esophagus without swallowing. Pressure records were obtained from one such subject, who aided the flow by reducing his intrathoracic pressure by inspiration against a closed glottis. During a 4-second period he imbibed 8 oz. of water without concomitant increase in intrapharyngeal pressure. The pressures at the cricoid level decreased intermittently and thus allowed water to pass into the esophagus. It appears therefore that the cricopharyngeus, with training, can be brought under voluntary control.

DISCUSSION

Our findings indicate that propulsion of the bolus through the pharynx in many ways resembles that through the esophagus. In both instances the bolus, if liquid, is injected from above under

pressure and traverses the lumen of the organ under this impetus. The wave of constrictor contraction, like the peristaltic wave in the esophagus, follows the bolus and sweeps along its last remains, but the whole process is much faster in the pharynx. It is of interest that Kronecker and Meltzer (1) in 1883 found two peaks on balloon records of deglutition pressures in the pharynx and postulated that the first represented the appearance of the bolus, and the second, constrictor contraction. Since that time most investigators have described a single positive wave. Between the pharynx and the esophagus lies a zone in which motor activity is completely different. Our investigations confirm that this has the characteristics of a sphincter. As shown by Fyke and Code (5), resting pressures in this zone are high. In addition, our radiographs indicate that at this point the lumen is constricted, and the position of the sphincter is in accordance with the view that it is the cricopharyngeus. This sphincter relaxes in a coordinated fashion just before the bolus, and contracts again after the bolus has gone by. Although the sphincter normally functions in a reflex fashion, it apparently may be relaxed or dilated voluntarily in certain subjects who have the faculty of pouring fluid into the esophagus without swallowing. Conversely, Dail, Affeldt, and Collier (13) have shown that patients with respiratory paralysis can be taught to keep the cricopharyngeus closed and the glottis open during air "swallowing," thus inflating the lungs (glossopharyngeal breathing).

As no pressure below resting intraesophageal pressure was found, these results refute the suggestion that suction is concerned in swallowing. Resting intraesophageal pressure usually is a few millimeters Hg below that in the pharynx, and relaxation of the cricopharyngeus establishes a mild downward gradient, but this factor would appear to be of negligible importance in propelling the bolus.

A somewhat different type of swallowing pressure complex was described by Fyke and Code (5). At the cricopharyngeal junction, they found markedly negative deglutition pressures. Considerably below the resting pressure level in the esophagus, this negative deflection was followed by a final positive wave. Our results indicate that the pressure changes in this region represent a tendency toward equalization of intraluminal pressure between the sphincteric zone and the esophagus. No pressures below resting esophageal levels occurred. In addition, in our experience the final positive wave was evident when it was likely that the tube had moved into the esophagus with the bolus. These discrepancies may be related to technical factors, in particular the difficulty that is experienced in maintaining the recording tip in a constant position in relation to the wall of the pharynx and esophagus during swallowing. In this connection, simultaneous radiography is of considerable value in detecting movements of the tube.

While it is not of importance in the normal mechanism of swallowing, suction can be used to facilitate the flow of liquids into the esophagus, as was the case in the subject who by relaxing his cricopharyngeus could pour fluids down. Here the larynx was elevated and closed off from the pharynx, but no impetus was imparted by the tongue or pharyngeal muscles. In certain dysphagias the cricopharyngeus may function normally, yet the pharyngeal constrictor muscles have lost their power (14). Voluntary relaxation of the cricopharyngeus, if it could be taught these patients, might help them in taking fluids.

SUMMARY

Resting intraluminal pressure was elevated at the pharyngo-esophageal junction in each of 30 normal subjects, the mean pressure being 35 mm. Hg. This zone of elevated resting pressure corresponded radiographically with the position of the cricopharyngeal sphincter.

During swallowing, intraluminal pressures were recorded simultaneously from three sites in the pharynx and esophagus while serial radiographs were taken at 12 per second. In the pharynx two peaks of pressure occurred, the first coinciding with the entry of the bolus and the second with constrictor contraction.

In the high pressure zone, at the cricoid level, an abrupt fall to, or to just above intraesophageal pressure occurred on swallowing, followed by an equally abrupt return to the high resting level after the passage of the bolus. These findings support the view that the cricopharyngeus is a true sphincter.

No pressures lower than esophageal pressures were recorded during the passage of the bolus; therefore, swallowing does not involve suction.

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THE DYNAMICS OF SWALLOWING. II. NEUROMUSCULAR DYSPHAGIA OF PHARYNX

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Neuromuscular disorders involving the pharyngeal phase of swallowing have hitherto been classified on a radiological basis into descriptive but rather nonspecific categories such as vallecular dysphagia (1), so called on account of the residuum of barium seen after swallowing in the valleculae and pyriform sinuses, spasm of the mouth of the esophagus (2, 3) and cricopharyngeal achalasia (4). The exact pathophysiology has been difficult to define using radiological techniques alone, although the development of cinefluoradiography in recent years has enabled detailed observation of the anatomical movements of deglutition. This technique, however, has as yet given little additional information about the nature and extent of the propulsive defect in the neuromuscular dysphagias. In particular, the question of whether functional disturbance of the cricopharyngeus [1] hinders the passage of the bolus is unsettled.

To investigate disorders of the swallowing mechanisms in patients with various neuromuscular dysphagias, we have applied the same technique of intraluminal pressure recording and simultaneous rapid serial radiography which has been used to investigate normal swallowing (5).

METHODS

With the patient supine, resting intraluminal pressures were recorded from the pharynx and esophagus by means of an open-ended, metal-tipped polyethylene tube attached to a Sanborn electromanometer. This was passed through the nose until its tip lay in the esophagus and then, under fluoroscopic control, withdrawn centimeter by centimeter. At each level, resting intraluminal pressure was recorded.

Intraluminal pressures on swallowing were recorded simultaneously by means of three such tubes fastened together so that the distance between the distal and middle tip was 2 cm. and between the middle and proximal tip 4 cm. Whilst pressures were being recorded during barium swallows, serial radiographs at a rate of 6 per second were taken in the anteroposterior and lateral planes. All swallowing studies were made with the patients in the sitting position, as many had difficulty in swallowing when supine.

Patients studied. Seven patients with dysphagia were studied (Table I). Four had had bulbar poliomyelitis, two suffered from myasthenia gravis, and the seventh had dystrophia myotonica. Six patients were studied by simultaneous radiography and pressure recording; only pressure records were obtained in the seventh patient, a 30-year-old male with mild postbulbar dysphagia.

Dysphagia following bulbar poliomyelitis

Resting intraluminal pressures. Pressures in the lumen of the pharynx and esophagus were within normal limits. At the level of the cricoid cartilage a zone of high resting pressure was found, resembling that present in normal subjects and caused, it is believed, by tonic contraction of the cricopharyngeus (5, 6). In the four patients, maximal resting pressures in this high pressure zone were 48, 25, 30, and 22 mm. Hg, respectively, figures which correspond well with the mean normal value of 35 mm. Hg. In terms of the pressures recorded, no evidence of either paralysis or hypertonicity of the cricopharyngeus was obtained.

Deglutition pressures (Figure 1). In three of the four subjects hardly any pressure change occurred in the upper or lower pharynx. The initial peak of intrapharyngeal pressure was never present, but in the lower pharynx a slight increase in pressure was occasionally seen at the time the second peak of pressure occurs in normal subjects. In the fourth subject, who had only mild

[1] The terms cricopharyngeus and cricopharyngeal sphincter are here used to include the entire sphincteric mechanism, composed of striated muscle, and including not only fibers from the inferior constrictor (*i.e.*, the cricopharyngeus) but perhaps also circular muscle from the upper esophagus.

TABLE I

Patients studied

Disease	Patient's sex and age	Duration of dysphagia	Disability at time of study
Dysphagia following poliomyelitis	Male 27	7 months	Nasal voice. Frequent choking on swallowing and occasional regurgitation through the nose.
	Female 32	9 months	Nasal voice. Had required tube feeding for several months after attack and even at time of study could only swallow liquids and semi-solids.
	Male 41	2 years	Nasal voice. Difficulty in swallowing solids.
	Male 30	4 months	Normal voice. Difficulty in swallowing solids towards end of meal.
Myasthenia gravis	Female 19	2 years	Nasal voice. Moderate dysphagia relieved by prostigmine.
	Male 65	3 months	Slightly nasal voice. Difficulty in swallowing partially relieved by prostigmine.
Dystrophia myotonica	Male 60	5 years	Nasal voice and dysarthria. Marked dysphagia. Swallowing had to be assisted by tilting head backwards.

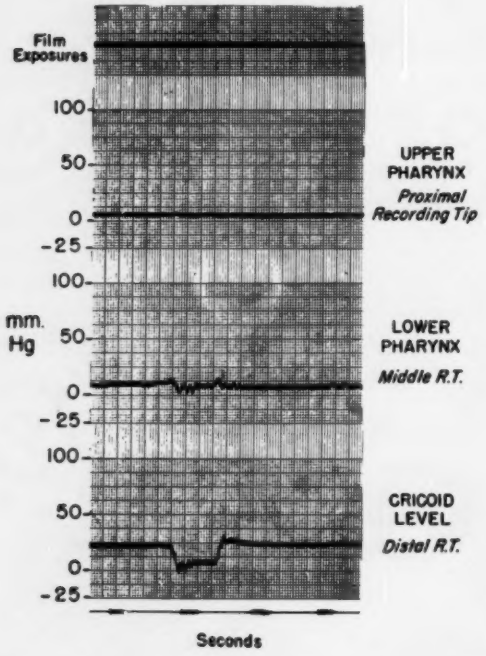

FIG. 1. DEGLUTITION PRESSURES IN A PATIENT WITH DYSPHAGIA FOLLOWING POLIOMYELITIS

R.T. = recording tip.

dysphagia, pressure complexes normal in type but low in amplitude were recorded from the pharynx.

The pressures recorded in the cricopharyngeal area showed a response to swallowing that was normal in configuration and in direction, degree and duration (less than 1.2 seconds) of change. Many swallows were not followed by pressure complexes in the cervical esophagus, but when these did occur, they were of normal configuration and amplitude.

Radiologic appearance and correlation with pressure changes (*Figures 2 and 3*). Essentially similar findings were obtained in all three patients. The tongue apparently functioned normally, propelling the bolus back into the pharynx. However, the soft palate never closed off the nasal passages, presumably one reason why pressure could not be generated in the upper part of the pharynx. The barium, once introduced into the pharynx, ran down the posterior aspect of the tongue into the valleculae and down the pyriform sinuses. Elevation of the larynx and closure of the glottis were seen but appeared late and only when the barium bolus was well into the pharynx. As the larynx rose, the cricopharyngeus opened to allow the barium to pass on into the esophagus. Following the abrupt closure of the cricopharyngeus, a resi-

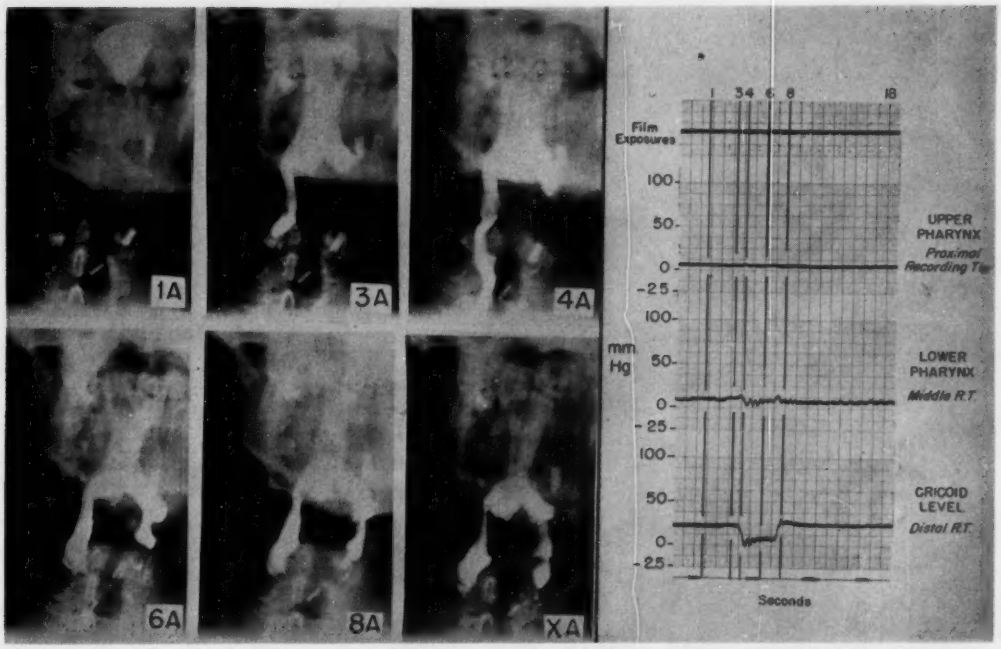

FIG. 2. SIMULTANEOUS RADIOGRAPHIC AND INTRALUMINAL PRESSURE STUDIES IN A PATIENT WITH DYSPHAGIA FOLLOWING POLIOMYELITIS

Plate XA was taken 10 seconds after swallowing and shows the barium residue in the pharynx.

due of barium was left in the valleculae and pyriform sinuses.

Comment. That the patient with dysphagia following bulbar poliomyelitis can develop little if any change in intrapharyngeal pressure on swallowing has been noticed in one case previously reported from this laboratory (7). This finding is in keeping with the extensive paralysis of the pharyngeal constrictor muscles and the muscles of the soft palate which is frequently found following bulbar poliomyelitis. It is remarkable that in the presence of this extensive paralysis of the pharyngeal constrictors the cricopharyngeus should remain intact and function normally. Sparing of the cricopharyngeus, however, has been noted radiologically in other bulbar disorders which have caused pharyngeal constrictor weakness (8). Possibly the differential effect of bulbar poliomyelitis on constrictors and cricopharyngeus is related to the innervation of the pharyngoesophageal area. The muscles of the upper pharynx are supplied directly from motor cells in the nucleus ambiguus, through the ninth and tenth cranial nerves. The esophagus derives its parasympathetic (motor) supply from the dorsal nucleus of the vagus via preganglionic fibers running in the vagus nerve. At what point in the gullet the dorsal nucleus takes over the motor nerve supply from the nucleus ambiguus, or to what extent these supplies overlap, is uncertain although Mitchell states that the field supplied by the dorsal nucleus extends as high as the lower pharynx (9).

Poliomyelitis involves the nucleus ambiguus much more frequently than it does the dorsal nucleus of the vagus, and Faber and Silverberg (10) could discover no evidence of damage to the latter at autopsy in patients with bulbar poliomyelitis. Our finding of sparing of the cricopharyngeus after bulbar poliomyelitis might thus be explained on the grounds that this area, unlike the pharyngeal constrictor muscles, is adequately supplied by nerves from the dorsal nucleus. Functionally, then, this sphincter behaves as part of the esophagus rather than the pharynx.

Although flaccid paralysis of the upper esopha-

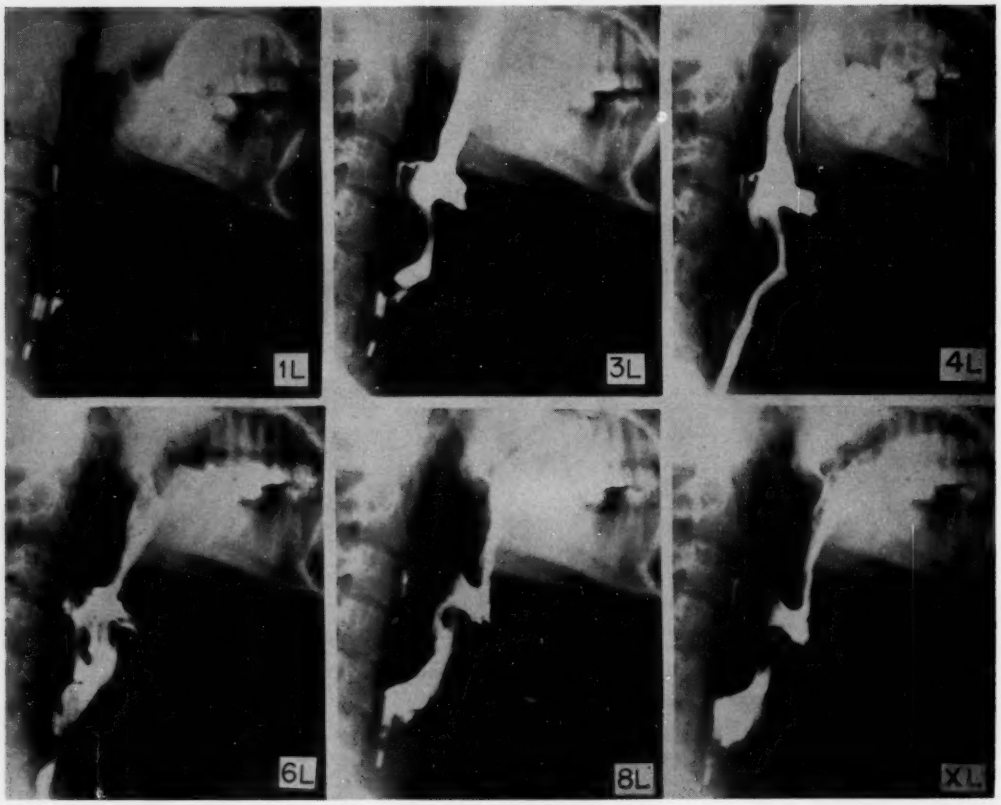

FIG. 3. DYSPHAGIA FOLLOWING POLIOMYELITIS
Lateral plates exposed at the same times as the anteroposterior films shown in Figure 2.

gus has been stated to occur in bulbar lesions (11), the evidence is not clear. The upper esophagus appeared normal in our patients, and normal swallowing pressures occurred, albeit infrequently. These observations are in keeping with the finding of normal complexes in the mid- and distal esophagus of a bulbar poliomyelitis patient with total dysphagia (7). It would appear that little disturbance of intrinsic esophageal motility is present in patients with dysphagia following bulbar poliomyelitis.

In the patients here reported, a major part of each bolus passed into the esophagus with the aid of gravity, even in the absence of normal propulsive forces. Some barium remained in the pharynx because the flaccid pharyngeal walls and absence of constrictor muscle activity allowed pools to remain in the pyriform sinuses and vallecular. Although the slow trickle of barium from the pharynx into the esophagus was interrupted by the closure of the cricopharyngeus, it seemed unlikely that a great deal more barium would have left the pharynx had this sphincter remained open. Certainly our findings failed to show any evidence of cricopharyngeal achalasia which has been described after poliomyelitis (4) or of spasm of the cricopharyngeus. Section of the cricopharyngeus has been used in the treatment of dysphagia following poliomyelitis (12). In light of our observations it seems doubtful whether this operation has any rational basis. We found no evidence of abnormal function of the cricopharyngeus, and the muscle can be relaxed again by taking a second "dry" swallow to allow the passage of any of the bolus that sphincteric closure might have prevented.

Myasthenia gravis

Two patients with myasthenia gravis were studied immediately before and 20 minutes after their regular dose of prostigmine was given.

Resting intraluminal pressures. Pressures in the lumen of the pharynx and esophagus were within normal limits. At the level of the cricoid cartilage, in contrast to the group with dysphagia following poliomyelitis, both patients showed a low resting pressure. In one patient, no elevation could be detected at all (Figure 4), and in the other, the maximum resting pressure attained was 15 mm. Hg. After prostigmine the resting pressure rose to 10 mm. Hg above the esophageal level in the first patient (Figure 4). In the second, the maximum resting pressure showed no change.

Deglutition pressures (Figure 4). On swallowing, both patients showed two very low peaks of intrapharyngeal pressure, corresponding in time with those seen in normal subjects. At the cricoid level, no pressure changes occurred in the patient who had no elevation of resting pressure; in the other patient, the swallowing complex was normal in configuration but slight in degree.

Radiologic appearance and correlation with pressure changes (Figure 5). The patients appeared to initiate swallowing by allowing the barium to run off the tongue into the pharynx. The normal thrusting action of the tongue by its approximation against the hard palate was not seen. As was true with the group with dysphagia following poliomyelitis, the barium had passed well into the pharynx before the larynx was elevated. In both subjects, as judged radiologically, the nasopharynx was closed off and weak constrictor muscle contractions occurred. In the patient with the high resting pressure at the cricopharyngeal area, the sphincter appeared to function normally.

After prostigmine, the thrusting action of the tongue returned sufficiently to give an impetus to the bolus and to cause a larger initial peak in the upper and lower pharyngeal records. The second peak corresponding with constrictor activity also showed a slight increase in amplitude. No change in cricopharyngeal activity was detectable radiologically, although the pressure records showed some return towards normal pattern.

Comment. The muscular disorder in the two patients with myasthenia gravis was more generalized than that which followed bulbar poliomyelitis. In addition to the pharyngeal constrictor muscles, the tongue and cricopharyngeus were weakened and may have contributed to the difficulty in swallowing. Nevertheless, in the two patients studied the palatal muscles appeared to retain sufficient function to close off the nasal passages tightly enough to allow some pressure to build up in the pharynx. Since myasthenia gravis is a disease of striated muscle, involvement of the cricopharyngeus would be expected.

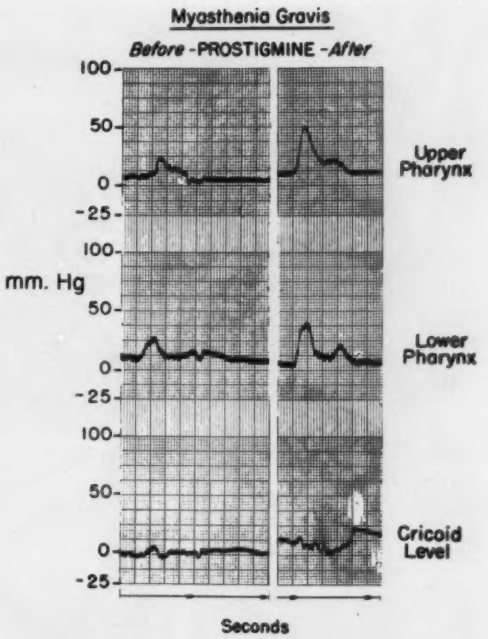

FIG. 4. INTRALUMINAL PRESSURE RECORDS FROM A PATIENT WITH MYASTHENIA GRAVIS SHOWING AN INCREASE IN AMPLITUDE AFTER PROSTIGMINE INJECTION

Our finding of increased intrapharyngeal deglutition pressures after prostigmine is in keeping with the improvement in swallowing noted radiographically after prostigmine by Schwab and Viets (13). They observed a reduction in the pharyngeal residue of barium in 17 out of 19 myasthenics given 1.5 mg. of prostigmine subcutaneously.

Dystrophia myotonica

Resting intraluminal pressures. Resting intraluminal pressures were in the normal range except

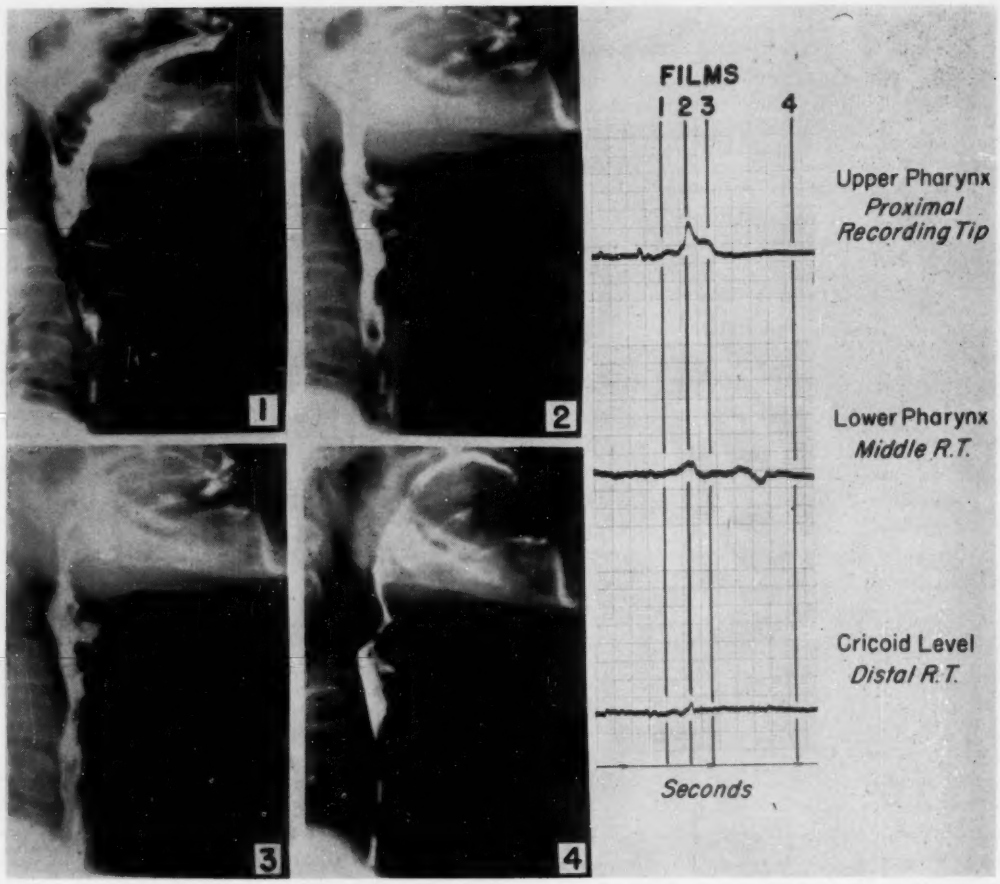

FIG. 5. SIMULTANEOUS RADIOGRAPHIC AND MANOMETRIC STUDIES IN A PATIENT WITH MYASTHENIA GRAVIS

for the cricopharyngeal area, where the normal elevation of resting pressure was not detected. In addition, the recording tip could be passed back and forth from pharynx into the esophagus without encountering a sense of resistance.

Deglutition pressures. Virtually no change in intraluminal pressures occurred on swallowing either in the pharynx, the cricopharyngeal zone or the upper esophagus.

Radiologic appearance and correlation with pressure changes. The patient had extensive involvement of the lingual muscles and could initiate swallowing only by tilting his head backwards and thus allowing the bolus to run into the pharynx. Radiologically, the thrusting action of the tongue was not seen and the barium appeared to run passively off the back of the tongue. It then trickled slowly through the pharynx. The nasal passages did not appear to be closed off, and very little elevation of the larynx could be observed. After swallowing, a considerable residue of barium remained in the recesses of the flaccid pharynx.

Comment. The swallowing disorder in this patient resembled that seen in myasthenia gravis in that all the muscle groups involved in the swallowing act appeared to be involved.

DISCUSSION

This study indicates that a number of factors may be responsible for difficulty in swallowing in patients with neuromuscular dysphagia. In nor-

mal swallowing the impetus given to the bolus by the action of the tongue pressing against the hard palate appears to be of great importance in propelling the bolus through the pharynx and esophagus. In neuromuscular dysphagia this impetus is often absent because of either paralysis of tongue muscles themselves or inability to close off the nasal passages, with dissipation of pressure and possibly regurgitation through the nose. Under these circumstances the bolus passes into the pharynx largely by the action of gravity, its speed is much reduced, and swallowing is often impossible in the supine position. The head of the bolus appears to pass well over the back of the tongue into the valleculae before the involuntary phase of swallowing begins. In relation to the first voluntary instigation of swallowing, consequently, the involuntary functions of laryngeal elevation and closure and of cricopharyngeal opening may occur late. Because of the slow rate of flow of the bolus, however, this delay is probably more beneficial than otherwise. The cricopharyngeus still opens in advance of arrival of the bolus, and closing of the respiratory passages is postponed until the last moment, thus shortening the long respiratory interruption necessitated by the slow passage of swallowed material.

Residues of barium are seen in the valleculae and pyriform sinuses in the presence of paralysis of the pharyngeal constrictors irrespective of whether the cricopharyngeus is functioning normally or is paralyzed. These residues therefore appear to be due to failure of the action of the constrictor muscles in emptying these recesses.

SUMMARY

Disorders of swallowing in four patients with post-poliomyelitis dysphagia, two with myasthenia gravis and one with dystrophia myotonica have been studied by the recording of intraluminal pressures from the pharynx and esophagus combined with synchronous rapid serial radiography during barium swallows.

After poliomyelitis, failure to close off the nasal passages, with consequent dissipation of oropharyngeal pressure, and paralysis of the pharyngeal constrictor muscles appeared to be the principal abnormalities. The cricopharyngeus functioned normally in each of the four patients, and no evidence of achalasia or spasm was obtained.

In two patients with myasthenia gravis and one with dystrophia myotonica, a generalized muscular weakness, particularly involvement of the tongue muscles, appeared to be important in producing dysphagia. The cricopharyngeus, a striated muscle, was involved.

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A HEPARIN-PRECIPITABLE FRACTION OF HUMAN PLASMA. I. ISOLATION AND CHARACTERIZATION OF THE FRACTION [1]

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Evidence has accumulated indicating that the fibrinoid deposits found in histopathologic material in certain human diseases—for example, acute lupus erythematosus and polyarteritis—may be derived, at least in part, from fibrinogen, fibrin, or a closely related protein (1, 2). Studies of the conditions under which similar deposits are laid down in the blood vessels of rabbits during the generalized Shwartzman reaction (3, 4) and after injection of gram-negative bacterial endotoxin in combination with acidic polymers of large molecular size (5, 6), have provided an experimental model for studying the origin of these deposits. In the rabbit a direct correlation between the disappearance of fibrinogen from the circulating blood and the appearance of fibrinoid within the glomerular blood vessels was observed, and the suggestion was made that endotoxin produced an increase in the *in vivo* precipitability of fibrinogen by acidic polymers (7, 8). It was shown that within less than one hour after an intravenous injection of endotoxin, a change in the stability of fibrinogen occurred which was manifested by the cold-insolubility of this protein in the presence of heparin (7, 9). It seemed possible that this heparin-precipitable form of fibrinogen might represent, in the rabbit, a precursor of fibrinoid, and a study of the occurrence of a similar cold-insoluble, heparin-precipitable component in human plasma was therefore undertaken.

This presentation is concerned with attempts to characterize the fraction.

Data will be presented which 1) describe the essential features necessary for the precipitation reaction to occur, 2) define the solubility in terms of pH, ionic strength, and a requirement for specific cations, and 3) establish a close relationship between the fraction and fibrinogen. The nature of the heparin-fibrinogen combination and its possible relationships to previously described fibrinogen fractions are discussed. Preliminary accounts of these observations have been presented elsewhere (10, 11).

MATERIALS AND METHODS

Heparin-precipitable fraction (HPF). In most of the experiments the precipitation reaction was studied utilizing the heparinized plasma of patients with various inflammatory diseases, in whose plasma prior measurements had detected relatively large amounts of the fraction. Single or pooled samples of such plasma containing a final heparin concentration of 0.1 mg. per ml. were employed unless otherwise stated.

For experiments in which the effect of various reagents upon the yield of precipitate from heparinized plasma was determined, the reagents were added directly to aliquots of the heparinized plasma prior to cooling to 2° C. The pH of heparinized plasma at room temperature ranged between 7.8 and 7.95. The plasma-reagent mixture was then chilled at 2° C for 18 hours. The resulting precipitate was separated by centrifugation in the International refrigerated centrifuge. It was washed twice with cold 0.05 M, pH 7.4 phosphate buffer and collected by centrifugation. The twice-washed precipitates were then dissolved in buffer at 37° C and the protein content was measured by the biuret technique (12) employing a Coleman Jr. spectrophotometer. This method is presented in more detail in the accompanying paper.

For experiments in which the solubility of isolated fraction at varying ionic strength and pH was determined, the twice-washed precipitates from heparinized plasma were dissolved in buffer solutions of various ionic strength and pH values at 37° C. The resulting solutions were then chilled at 2° C for an additional 18 hours. Solubility was determined by measuring the protein content of the precipitate.

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[3] Established Investigator of the Minnesota Heart Association.

Anticoagulants. Heparin used for most of these studies was a solution of commercial heparin containing 10 mg. per ml. A purified sodium heparinate preparation (Upjohn, lot No. DX-629) was used in some experiments. Sodium citrate was employed at a final concentration of 0.4 per cent. Disodium ethylenediamine tetraacetate ("Versenate") as a 0.05 molar solution was used at the ratio of 0.5 ml. for each 5 ml. of blood. Solutions of sodium polymannuronic acid sulfate, "1148A," chitin sulfate, alginic acid sulfate, containing 10 mg. per ml. were used at the ratio of 0.1 ml. for each 5 ml. blood. These polymers were obtained through the courtesy of the Upjohn Company, Kalamazoo, Michigan. Chondroitin sulfate was generously supplied by Dr. G. Berenson, New Orleans.

Clottability was determined by adding thrombin to a mixture containing 2.0 ml. of the twice-washed fraction dissolved in pH 7.4, 0.05 M phosphate buffer, and 5 ml. phosphate-saline buffer. Since maximal clotting was produced by adding 30 units of thrombin, this amount was used. The weight of the clot was then determined, and the result expressed as per cent of total protein. No correction for occlusion (13) of other proteins was made.

Electrophoresis was performed by the zone method on paper, utilizing a Spinco Model R, paper strip apparatus, and pH 8.6, ionic strength 0.1 veronal buffer. To assure maximum solubility of the fraction, the chamber of the electrophoresis apparatus was placed in an incubator at 37° C for most of these studies. The fraction was isolated as described above, and redissolved at 37° C in veronal buffer. Ten microliters of the solution was then applied immediately to the paper strips. Most runs were made at 15 milliamps for 6 hours. The strips were heat-fixed and stained with bromphenol blue.

Ultracentrifugation studies were performed in collaboration with Dr. D. R. Briggs, of the Department of Agricultural Biochemistry of the University of Minnesota. All runs were made in a Model E Spinco analytical ultracentrifuge at 59,780 revolutions per minute at room temperature. Sedimentation constants (S_{20_w}) were calculated by standard methods. The fraction was prepared for these studies as for electrophoresis, except that it was dissolved in 0.5 M NaCl at pH 7.4.

RESULTS

General observations

Heparinized plasma obtained from a patient with acute rheumatic fever or other acute inflammatory disease, when placed at 2° C for 18 hours, yields a coarse precipitate. Within approximately 20 minutes after chilling the plasma an opalescence appears; a precipitate then separates and settles out within a 3 to 6-hour period. The precipitates vary somewhat in texture, consisting at times of heavy floccules, but on other occasions having a fibrillar appearance. Warming to 37° C for a few minutes redissolves the precipitate. No precipitation is observed at 18 hours in serum, or in citrated, oxalated, or versenated plasma (Table I [A]). However, when heparin is added to ci-

TABLE I

Precipitation occurring in plasma at 2° C with various anticoagulants

Anticoagulant*	Precipitation after 18 hours at 2° C gm. %
(A) Calcium binding agents	
Disodium ethylenediamine tetraacetic acid, 0.1 mM/ml.	0
Sodium citrate, 0.05 mM/ml.	0
Ammonium and potassium oxalate mixture, dry	0
(B) Heparin plus calcium binding agent	
Serum; 0.2 mg./ml. sodium heparin added before chilling	0
Sodium citrate, 0.05 mM/ml.; sodium heparin, 0.2 mg./ml. added before chilling	0.30
Disodium ethylenediamine tetraacetic acetate, 0.1 mM/ml.; sodium heparin, 0.2 mg./ml., added before chilling	0.34
(C) Sulfated polymers	
Sodium heparin, 0.2 mg./ml.	0.46
Sodium polymannuronic acid sulfate, 0.2 mg./ml.	0.41
1148A, 0.2 mg./ml.	0.56
Alginic acid sulfate, 0.2 mg./ml.	0.29
Chitin sulfate, 0.2 mg./ml.	0.58
Sodium citrate, 0.05 mM/ml. plus chondroitin sulfate, 0.2 mg./ml.	0.20

* Blood from an acutely ill patient was drawn into tubes containing the final concentration of anticoagulant indicated. The amount of precipitate in the plasma was measured after 18 hours at 2° C.

TABLE II

Influence of heparin concentration on cold precipitation from plasma

	Precipitate gm. %						
	Heparin concentration, mg./ml. whole blood						
Patient*	0.01	0.05	0.075	0.10	0.15	0.20	0.50
A	0.11	0.30		0.37		0.20	0.19
B		0.53	0.60	0.64	0.38	0.24	0.19
C		0.10	0.16	0.16	0.15	0.07	

* Five ml. of blood was taken from the patient and placed in tubes containing heparin in 0.1 ml. to give the final concentration indicated. The precipitate formed after 18 hours at 2° C was measured.

trated or versenated plasma precipitation occurs (Table I [B]), indicating that the component is present in decalcified plasma, but soluble at 2° C unless heparin is added.

Multiple washings of the precipitate from heparinized plasma with pH 7.4 phosphate buffer at 2° C do not significantly alter its solubility. It appears, therefore, that heparin combines with the plasma component forming a complex, insoluble at 2° C, and that precipitation does not occur as a result of changes in the solvent on addition of heparin.

Other heparin-like anticoagulant polysaccharides, such as alginic acid sulfate, chitin sulfate, 1148A, and polymannuronic acid sulfate, also produced a cold-precipitate which, although varying in amount, did not differ qualitatively from that observed in heparinized plasma (Table I [C]). Chondroitin sulfate, when added to citrated plasma, resulted in some cold-precipitation. Twice-washed precipitates added to washed RBC produced rouleaux formation regularly, and when added to normal oxalated blood greatly accelerated the erythrocyte sedimentation rate.

The effect of heparin concentration on HPF solubility in plasma

The amount of precipitation occurring in the plasmas of acutely ill patients containing varying amounts of heparin is illustrated in Table II. The data show a narrow range of heparin concentration at which maximum precipitate yield is obtained. The optimal concentration for maximum precipitate yield in this and similar experiments was in the range of 0.05 to 0.15 mg. heparin per ml. of whole blood.

TABLE III

*Effect of ionic strength on precipitation from heparinized plasma**

Ionic strength $\Delta\mu$	Precipitate gm. %
None	0.60
0.017	0.59
0.043	0.43
0.085	0.08
0.171	0.06

* To 1.8 ml. heparinized plasma taken from an acutely ill patient, 0.2 ml. of an NaCl solution was added to give the final added Δ ionic strength indicated. After 18 hours at 2° C the amount of precipitate in these plasmas was determined.

TABLE IV

Effect of pH on precipitation from heparinized plasma

Final pH of plasma*	Precipitate gm. %
7.9 (untreated)	0.50
7.5	0.64
7.0	0.68
6.7	0.50

* To heparinized plasma taken from an acutely ill patient, 0.1 N HCl was added in amounts required to give indicated final pH value measured at room temperature. After 18 hours at 2° C the amount of precipitate in the plasmas was determined.

The effect of ionic strength and pH on HPF solubility

The effect of ionic strength and pH alterations of heparinized plasma on precipitate yield is illustrated by a typical experiment shown in Table III. In these experiments various concentrations of NaCl were added to plasma prior to chilling. The yield of HPF decreased progressively with increasing ionic strength.

The desired final pH of heparinized plasma was obtained by stepwise addition of 0.1 N hydrochloric acid. In an illustrative experiment recorded in Table IV, final pH values at room temperature were 6.7, 7.0, 7.5, and 7.9. Maximum precipitate yield from plasma after chilling to 2° C for 18 hours was obtained at pH 7.0.

The effect of varying ionic strength at several pH values on the solubility of the isolated fraction was then examined. Table V, illustrating the data obtained in four identical experiments, shows that the isolated fraction, similar to the fraction

TABLE V

Effect of pH and ionic strength on solubility of isolated heparin-precipitable fraction

Ionic strength*	Precipitate, gm. % pH 6.7	pH 7.0	pH 7.4	pH 7.7
0.05	0.33	0.32	0.32	0.31
0.20	0.20	0.15	0.08	0.04
0.35	0.03	0.01	0	0
0.50	0.02	0	0	0

* Aliquots of twice-washed cold-precipitates from the pooled heparinized plasma of four acutely ill patients were dissolved at 37° C in phosphate buffers of the indicated pH and ionic strength. After 18 hours at 2° C the amount of precipitate was measured.

TABLE VI

Effect of calcium and magnesium ions on precipitation from bivalent ion-depleted, heparinized plasma

Treatment of plasma*	Precipitate gm. %
a. Heparinized plasma, untreated	0.30
b. Bivalent ion-depleted heparinized plasma plus $CaCl_2$ (final concentration, mM/ml.)	
0.000	0.19
0.002	0.20
0.003	0.27
0.004	0.29
0.005	0.30
0.01	0.40
c. Same plus $MgCl_2$ (final concentration, mM/ml.)	
0.005	0.31
0.01	0.40

* Heparinized plasma was pooled and divided into aliquots. Na-cycled Dowex 50 was added to the plasma to remove bivalent ions. To 1.8 ml. of this plasma was added 0.2 ml. of the reagent indicated to give the final concentration shown, and the precipitate determined after 18 hours at 2° C.

in plasma, increases in solubility with increasing ionic strength. The limits of this "salting in" effect are not shown in this experiment. In other experiments it was found that the isolated fraction could be precipitated by high concentrations of NaCl.

The effect of calcium and magnesium ions on precipitation from heparinized plasma

As shown in Table I (B), decalcified heparinized plasma yielded less precipitate than heparinized plasma alone. Since decalcification by treating the plasma with sodium-cycle Dowex 50 also reduced the amount of precipitate in heparinized plasma, it appeared that this effect was not due to the increased ionic strength provided by the decalcifying agents, but to binding of bivalent cations. The effect of replacing calcium or magnesium ions on HPF yield from plasma depleted of these cations was therefore investigated.

In the experiment summarized in Table VI, addition of calcium chloride to a final concentration between 0.004 and 0.005 millimol per ml. resulted in a yield equivalent to that obtained from the original heparinized plasma, within the limits of experimental error. Calcium added to the cation-depleted plasma to 0.01 millimol per ml. resulted in a larger yield of precipitate than that occurring in the untreated plasma. The addition of magnesium ions to depleted plasma resulted in similar increases in precipitation.

Evidence for a relationship between the heparin-precipitable fraction and fibrinogen

In previous studies evidence that the heparin-precipitable fraction appearing in the plasma of endotoxin-treated rabbits was closely associated with or related to fibrinogen, depended upon a) absence from serum, b) partial clottability by thrombin, c) similar electrophoretic mobility, and d) disappearance *in vivo* along with circulating fibrinogen when high molecular weight acidic polymers having the property of precipitating fibrinogen *in vitro* were administered to endotoxin-treated rabbits (7, 9). Inasmuch as the fraction was not demonstrable in normal rabbit plasma, appearing only after injection of bacterial endotoxin, it was concluded that the fraction differed qualitatively from fibrinogen (7).

Evidence that the heparin-precipitable fraction from humans resembles fibrinogen has been sought. As indicated in Table I, the component is absent from the heparinized serum of patients whose heparinized plasma yields large amounts of precipitate. This and many similar negative observations on samples from ill patients indicate clearly that these precipitates are not cold-precipitating globulins of the type reported by Wintrobe and Buehl (14), Watson and Lerner (15), and others.

The clottability of the isolated fraction obtained from acutely ill patients varied between 41 and 63 per cent of the precipitable protein, as shown

TABLE VII

Clottability of heparin-precipitable fraction

Patient	Precipitate* gm. %	Clottable protein %
A	0.73	41
B	0.57	44
C	0.54	61
D	0.43	44
E	0.27	63
F	0.04	50

* Twice-washed precipitates from heparinized plasma were redissolved in pH 7.4 buffer and thrombin. The clottable protein is given as per cent of precipitable protein.

in Table VII. Interpretation of these data is complicated by the antithrombic effect of heparin present in the complex; it can be concluded only that these represent minimum values for clottability.

The technique of zone paper electrophoresis at 37° C has been used to examine precipitates from a number of acutely ill patients. A typical pattern, shown in Figure 1, compares migration of the fraction after 6 hours at 37° C with heparinized plasma and heparinized serum run simultaneously from the same patient. It may be seen that the fraction migrates as a single band at approximately the same rate as fibrinogen. However, the precise mobilities have not as yet been determined, because of difficulties inherent in free electrophoresis at or near room temperature (16).

Sedimentation of the twice-washed and redissolved fraction determined in the analytical ultracentrifuge at 20° C shows two peaks. A typical pattern is shown in Figure 2. The slower moving peak, accounting for approximately 80 per cent of the protein in most patients studied has a sedimentation constant (S_{20_w}) ranging between 7.9 and 8.3. This value is similar to that reported for human fibrinogen (17). The fast moving component has a sedimentation constant (S_{20_w}) varying between 10.2 and 12.5.

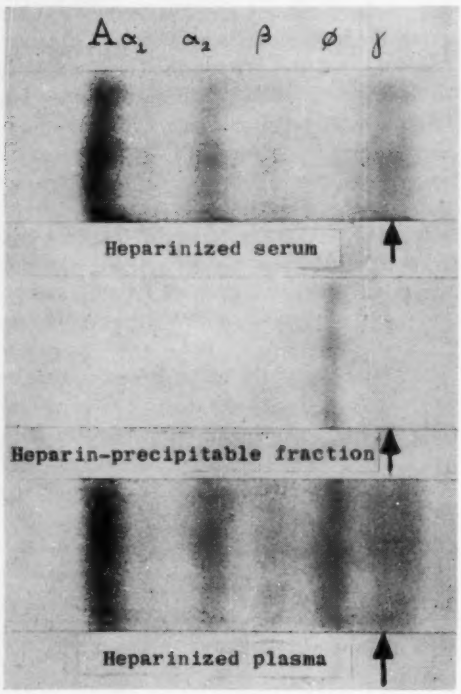

FIG. 1. ELECTROPHORETIC MIGRATION OF SIMULTANEOUSLY OBTAINED HEPARINIZED PLASMA, HEPARINIZED SERUM, AND HEPARIN-PRECIPITABLE FRACTION ON PAPER AT 37° C, IN pH 8.6 VERONAL BUFFER

Origin at arrow. The heparin-precipitable fraction migrates parallel to fibrinogen in the heparinized plasma.

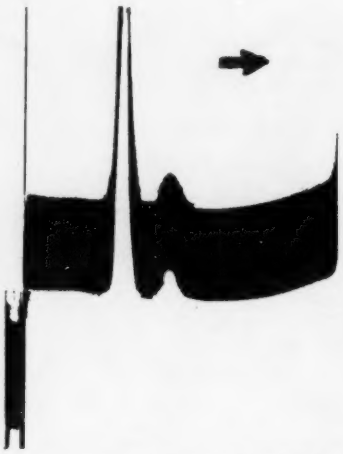

FIG. 2. SEDIMENTATION PATTERN OF HEPARIN-PRECIPITABLE FRACTION OBTAINED FROM AN ACUTELY ILL PATIENT

Forty-eight minutes run at 28° C in 0.5 M NaCl at 59,670 rpm in a Spinco Model E ultracentrifuge. The slower peak has sedimentation constant $S_{20_w} = 7.9$; the faster peak, $S_{20_w} = 12.5$.

TABLE VIII

Influence of heparin on cold-solubility of isolated fibrinogen

Determination*	Red Cross Lot 47	Red Cross Lot A50	Cutter Lot E4530
Total protein (*gm.* %)	1.9	1.85	1.1
Clottable protein (% of total protein)	56.4	78.0	64.0
Heparin, cold-insoluble protein (% of total protein)	11.2	21.6	25.4
Cold-insoluble protein (% of total protein)	2.7	3.8	5.4

* Solutions of the dried preparations were made up to the protein concentration indicated; the clottability was determined, and remainder of the solution divided into two portions; to one aliquot, heparin, 0.2 mg. per ml., was added; after 18 hours at 2° C the protein content of the twice-washed precipitates was determined.

Influence of heparin on solubility of isolated human fibrinogen

Heparin, 0.2 mg. per ml., was added to aliquots of two lots of outdated Red Cross purified human fibrinogen, and one lot of Cutter human fibrinogen, to determine its effect on cold-solubility of the isolated protein. Other aliquots without heparin were examined simultaneously. The results of this experiment, given in Table VIII, indicate that after standing 18 hours at 2° C, 11.2 and 21.6 per cent of the protein in the Red Cross fibrinogen precipitated, whereas 25.4 per cent of the other preparation was insoluble. Small amounts of cold-insoluble material precipitated without added heparin. It is clear from this experiment that the bulk of protein in these preparations is not insoluble in the presence of heparin at 2° C, although up to 78 per cent is clottable.

DISCUSSION

These experiments define some of the attributes of a component of human plasma characterized by a low solubility in the presence of heparin or related acidic polymers. Evidence has been presented that the plasma component is present, but soluble in freshly drawn decalcified plasma. Addition of heparin decreases the solubility of the fraction so that it is demonstrable simply by chilling to 2° C. The solubility of the isolated precipitable fraction is lowest in the presence of bivalent cations, at low temperature, low pH, and low ionic strength. In its solubility properties, absence from serum, clottability, electrophoretic mobility, and sedimentation characteristics, human HPF appears, as in the case of rabbit HPF (7), to be very similar to fibrinogen. On the other hand, experiments in which heparin was added to "purified" fibrinogen preparations indicate that only a small percentage of the protein in fibrinogen preparations used for clinical purposes is insoluble at low temperatures in the presence of heparin.

Considerable evidence has accumulated (18–22) indicating that certain acidic polymers have a strong affinity for fibrinogen and the beta lipoproteins, less for other proteins, and that the complexes apparently formed between them exhibit solubility properties and resistance to denaturation not characteristic of the native protein. Astrup and Piper (18) apparently were first to recognize and study such complexes systematically. They found that cellulose trisulfuric acid, polyanethol sulfonate, and similar high molecular weight compounds, precipitated fibrinogen from citrated plasma or purified fibrinogen preparations at room temperature. The effects of polymer concentration, pH, ionic strength, and magnesium ions on this reaction were similar to those described above for cold-precipitation from heparinized plasma. They reported no precipitation occurring with chitin sulfate or heparin; however, their experiments were conducted at room temperature. Experiments not included in this paper confirm their observations with polyanethol sulfonate.

Ricketts (19), Walton (20, 21), and others (22) more recently have examined the interaction between dextran sulfates of varying molecular weight and fibrinogen. These studies have shown that dextran sulfates of intrinsic viscosities 0.24 to 0.4 added to fibrinogen solutions result in precipitation at 4° C but not at 37° C. Dextran sulfates of higher intrinsic viscosity and presumably higher molecular weight, however, precipitated fibrinogen at 37° C. Recently Kerby (23) reported that chondroitin sulfate added to citrated plasma from patients with inflammatory disease resulted in cold-precipitation of a polysaccharide-protein complex.

These various items of evidence indicating the affinity of high molecular weight sulfated polysaccharides for fibrinogen permit speculation on a possible physiological role of the protein which precipitates with heparin, as HPF, *i.e.*, in the removal from the circulation of high molecular weight polysaccharides that arise from damage to ground substance. Such protein-polysaccharide complexes, if aggregated into macromolecular particles of sufficient size, would be immediately subject to removal by the cells of the reticuloendothelial system. Should the rate of complex formation exceed the capacity for removal by the R. E. system, deposition of the complexes, as fibrinoid, might occur. The experimental data available (24–26) lend support to this possibility.

Some properties of HPF closely resemble those of Fraction I-1, a cold-insoluble plasma component, described by Morrison, Edsall, and Miller (27), and more recently investigated by Edsall,

Gilbert, and Scheraga (28). These workers found that Fraction I-1 was insoluble at 4° C at low pH, low ionic strength in the presence of 0.5 per cent ethanol but soluble at 20° C. Like HPF, Fraction I-1 was up to 50 per cent clottable, and upon ultracentrifugal analysis was resolved into two components. The more abundant component had a sedimentation constant of 7 to 8.4 S, that of purified fibrinogen; the minor component sedimented more rapidly with a constant from 9.7 to 12.2 S. These values are similar to those obtained with HPF samples. Further studies (28) of the non-clottable fraction representing the residue of Fraction I-1 remaining after clotting with thrombin suggested that this was a globulin closely related to fibrinogen, perhaps a dimer. The relationship of the non-clottable fraction of HPF to this non-clottable globulin requires further investigation. A comparison of the N-terminal amino acids of HPF, fibrinogen, fibrin, and cold-insoluble globulin is being made currently in this laboratory.

Three other fibrinogen-like proteins having solubility properties which resemble HPF have been described previously. Morrison (29) studied a component designated "contractinogen" obtained from the plasma of pregnant or acutely ill patients by precipitation with 16⅔ per cent saturated ammonium sulfate. Contractinogen was reversibly cold-insoluble and showed an unusual propensity to clot spontaneously, and then retract markedly. This protein, rather than the more soluble clottable fractions, was thought to be responsible for aggregation and settling of red blood cells in the determination of the erythrocyte sedimentation rate.

Another fibrinogen component, studied extensively by Lyons (30) and termed "fibrinogen B," has been characterized by its insolubility when combined with certain naphthoquinone derivatives. "Fibrinogen B" was reported to be present in significant amounts in a varied assortment of diseases, particularly infectious and thrombotic states, but not in normal individuals.

Recently, Korst and Kratochvil (31) reported a case of pulmonary malignancy and multiple thromboses associated with "cryofibrinogenemia." A component from this patient's plasma was found to be reversibly precipitable at low temperatures, clottable, rouleaux-promoting and betanaphthol-precipitable. Heparin augmented cold-precipitation of the component from citrated plasma (32). The sedimentation value for the major component of this fraction ($S_{20_w} = 7.9$) resembled fibrinogen (17); a minor component had an S_{20_w} value of approximately 12 (estimated from published data). This "abnormal" fibrinogen resembles HPF in several ways, then, but apparently does not require the presence of heparin to precipitate at low temperatures. However, the possibility that an endogenous heparin-like component may have been present in their patient's circulation was not excluded. Where data are available, then, the close similarity of each of these "abnormal" fibrinogen fractions is striking.

Morrison (29) has suggested that contractinogen represents a qualitative alteration of fibrinogen, perhaps partial polymerization, occurring as a result of disease. A similar hypothesis was proposed in relation to the occurrence of HPF in experimental animals following endotoxin administration (7, 8). HPF in humans might also represent a polymer formed during the conversion of fibrinogen to fibrin, resembling those studied by Apitz (33), more recently by Donnelly, Laskowski, Notley, and Scheraga (34) and Casassa (35).

On the other hand, the possibility is not excluded that this group of fractions related to or associated with fibrinogen might represent a segment of the spectrum of fibrinogen-like proteins, each of which differs slightly from "pure" clottable protein, in solubility, and possibly in physiological function. Current studies of the relative turnover rates of this more insoluble fraction compared with the clottable fraction may illuminate this point.

SUMMARY AND CONCLUSION

Some of the physical and chemical properties of a fraction of human plasma which is characterized by cold-insolubility in the presence of heparin have been described. The data indicate that the fraction is a fibrinogen-like protein loosely combined with heparin, the resultant complex having altered solubility properties. Solubility of the fraction was found to be favored by increasing ionic strength, elevated temperature, alkaline pH, and the absence of calcium or magnesium ions. The amount of cold-insoluble fraction obtained from a given plasma is a function of the heparin

concentration. Evidence was obtained that the heparin-precipitable fraction is closely related to or associated with fibrinogen in that it is partially clottable, has similar mobility on electrophoresis, and is absent from serum. In the ultracentrifuge two components were found, the major fraction resembling fibrinogen, the minor component resembling the cold-insoluble, non-clotting component of Fraction I-1.

The possible relationships between heparin-precipitable fraction and previously reported fibrinogen-like proteins are discussed, and available data bearing on the possible nature and origin of the fraction are reviewed.

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A HEPARIN-PRECIPITABLE FRACTION OF HUMAN PLASMA. II. OCCURRENCE AND SIGNIFICANCE OF THE FRACTION IN NORMAL INDIVIDUALS AND IN VARIOUS DISEASE STATES[1]

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The preceding paper (1) describes the isolation and some of the physical chemical characteristics of a fraction of human plasma which precipitates at low temperatures in the presence of heparin. Evidence was presented suggesting that the fraction is closely related to or associated with fibrinogen. This paper is concerned with an account of the method routinely employed for estimating the fraction, and with a presentation of data obtained by this method in normal individuals of all ages, and serially in ill patients. Attempts have been made to correlate the changes in the component with other recognized measurements of inflammatory disease.

MATERIALS AND METHODS

Method of estimating the heparin-precipitable component. In earlier studies (2) of the heparin-precipitable fraction (HPF), observations were recorded by gross estimation of the amount and density of precipitates appearing in appropriately heparinized plasma after it had remained at 2° C for approximately 18 hours. Based upon preliminary experiments, an empirical method of measuring the precipitate was developed early in this investigation. Subsequent studies, presented in detail in the preceding paper, have suggested a number of possible improvements in this technique. However, in order that uniform observations could be made, the procedure described in detail below was adopted and employed unchanged throughout the investigation.

Five ml. of venous blood was taken from the fasting patient into a sterile rubber-stoppered glass tube containing 0.1 ml. heparin solution[3] (0.1 ml. commercial heparin containing 10 mg. per ml. or 100 units per ml. of sodium heparin) and mixed gently by inversion. After centrifugation at room temperature (2,500 rpm for 15 to 20 minutes) 2.0 ml. of supernatant plasma was pipetted into a sterile, acid-cleaned glass tube (14 × 100 mm.), covered, and the tube placed in a cold room maintained at 2° C. The pH at room temperature of plasmas after these manipulations was usually between 7.8 and 7.9. All subsequent operations during centrifugation and washing of the precipitates were carried out at 2° C. After standing 16 to 18 hours at this temperature, the tubes were centrifuged at 2,200 rpm for 10 minutes in an International refrigerated centrifuge at 2° C, the supernatant plasma poured off, and 3.0 ml. cold 0.05 M, pH 7.4 phosphate buffer was added. The precipitate was mixed by thumping the tube repeatedly with the index finger. After recentrifugation the supernate was decanted and the tube inverted to drain for 5 minutes; the precipitate was then rewashed with an additional 3.0 ml. of cold buffer. After this second wash, 1.5 ml. of the buffer was added to the tube, and the tube placed in a water bath at 37° C for 30 minutes. The precipitates rapidly went back into solution. One and five-tenths ml. of biuret reagent (3) was added to the redissolved precipitate, and after 30 minutes the optical density read against an identically run buffer blank at 555 mμ in a Coleman Jr. spectrophotometer. The protein content of the precipitate from 2.0 ml. of plasma was calculated from the optical density by reference to standard curves and expressed as grams per 100 ml. plasma. The standard was a solution of crystalline bovine serum albumin, the protein content of which was determined by the micro-Kjeldahl method (4). Results of replicate determinations on pooled plasma samples agreed within ± 0.02 gm. per cent by the method.

Fibrinogen determination. Plasma fibrinogen was estimated by a modification of the methods of Saifer and Newhouse (5) and Morrison (6) using oxalated plasma.

Two ml. of plasma was placed in a Petri dish containing 5.0 ml. of pH 6.3 phosphate-saline buffer solution.

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[2] Work performed as Helen Hay Whitney Foundation Research Fellow, and as Senior Investigator, Arthritis and Rheumatism Foundation.

[3] Heparin employed for many of these studies was generously supplied by the Upjohn Company, Kalamazoo, Michigan.

Four-tenths ml. of a freshly prepared thrombin solution in 0.9 per cent NaCl (8 mg. Parke-Davis Thrombin, Topical per ml. saline) was then added with gentle swirling to complete mixing. The clot which had formed after standing one hour at room temperature was washed twice with 0.9 per cent NaCl and twice with distilled water. After washing, the clot was collected, dried at 90° C for 18 hours, and weighed. A factor of 0.83 was applied to correct for occlusion of other plasma proteins, as suggested by Saifer and Newhouse (5), and the final result expressed as grams per 100 ml. plasma. Replicate determinations by this method agreed within ± 0.02 gm. per cent.

Erythrocyte sedimentation rate (ESR). The method of Westergren was employed throughout, using dry "double-oxalate" as the anticoagulant. The values given represent the fall in mm. in one hour at room temperature.

C-reactive protein (CRP). CRP was determined on serum specimens by the method described by Anderson and McCarty (7) using C-reactive protein antiserum (CRPA) kindly supplied by Schiefflin and Company.

Muco proteins (M.P.T.). These were determined on serum specimens by the method of Winzler, Devor, Mehl, and Smythe (8), and the results expressed as mg. mucoprotein tyrosine per 100 ml. serum.

Clinical material. Patients on the pediatric and medical services of the Variety Club Heart Hospital, in the Children's Rheumatism Clinic of the Heart Hospital, and the general pediatric services of the University of Minnesota Hospitals provided the bulk of clinical material utilized in these studies. A number of selected patients from surgical and obstetrical services were also studied. Serial studies were obtained wherever possible, specimens being taken for HPF, fibrinogen, ESR, CRP, and mucoproteins simultaneously at least twice a week, or as often as the condition of the patient indicated. Graphic charts prepared for each patient were studied in an attempt to correlate serial changes in clinical and routine laboratory findings with the parameters under examination. Representative charts are presented in the text.

Values for healthy individuals were obtained from three sources. The youngest group consisted of children between 2 and 15 years of age undergoing routine precamp physical examinations. The second group, varying in age from 16 to 45 years, was composed of students and faculty of the University of Minnesota undergoing routine physical examinations in the Student Health Service. The third group comprised adults, 46 to 75 years of age, who are being screened by extensive physical and laboratory examinations as a part of a cancer detection study. The author is indebted to Dr. Albert Sullivan of the Department of Surgery, University of Minnesota, for his cooperation in obtaining these specimens. All of the individuals in each group were afebrile, asymptomatic, and had no significant abnormalities on physical examination.

Statistical methods. For analysis of the data, the usual methods have been employed to determine the standard deviation and standard error. Dr. Jacob Bearman of the Department of Biostatistics of the School of Public Health, University of Minnesota, gave valuable advice on statistical analysis of the data, and calculated the regression coefficients.

RESULTS

HPF levels in healthy individuals

Levels of heparin-precipitable fraction (HPF) and fibrinogen were determined in 506 healthy individuals. These data are presented in three categories, consisting of individuals from 2 to 15, 16 to 45, and 46 to 75 years of age. The frequency distribution of values for male and female patients in each of the three groups is given in Figure 1. It may be seen that the distribution of values for the two older groups of individuals is somewhat skewed toward the higher values. This variation from Gaussian distribution, most striking in the middle age group, reflects, in part at

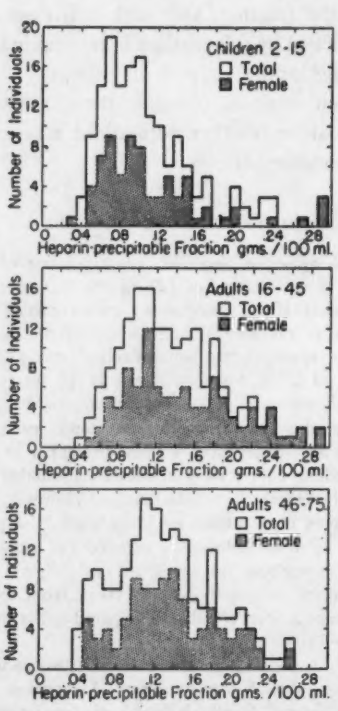

FIG. 1. FREQUENCY DISTRIBUTION OF NORMAL HPF VALUES IN THREE AGE GROUPS STUDIED

The total area in the distribution represents values for both males and females. The shaded area represents values for females of that distribution.

TABLE I

Heparin-precipitable fraction and fibrinogen values in normal persons, by age group and sex

Age group	Sex: Male Number	Male Mean gm. %	Male Standard deviation	Male Standard error mean	Female Number	Female Mean gm. %	Female Standard deviation	Female Standard error mean
			(A) Heparin-precipitable fraction					
3–15	69	0.101	0.043	0.005	70	0.104	0.042	0.005
16–45	80	0.129	0.049	0.006	99	0.144	0.059	0.006
46–75	85	0.121	0.051	0.006	103	0.137	0.053	0.005
			(B) Fibrinogen					
3–15	55	0.219	0.038	0.005	72	0.227	0.043	0.005
16–45	90	0.199	0.046	0.005	101	0.210	0.046	0.005
46–75	85	0.243	0.056	0.006	94	0.229	0.066	0.007

least, the higher range of values obtained for females. Values for the youngest group fell into a narrower range, and values for male and female patients showed similar distribution.

Table I A summarizes the mean values for HPF by age group and sex. The mean HPF values for males and females differed slightly but significantly ($P = < 0.001$) in the two older groups. The

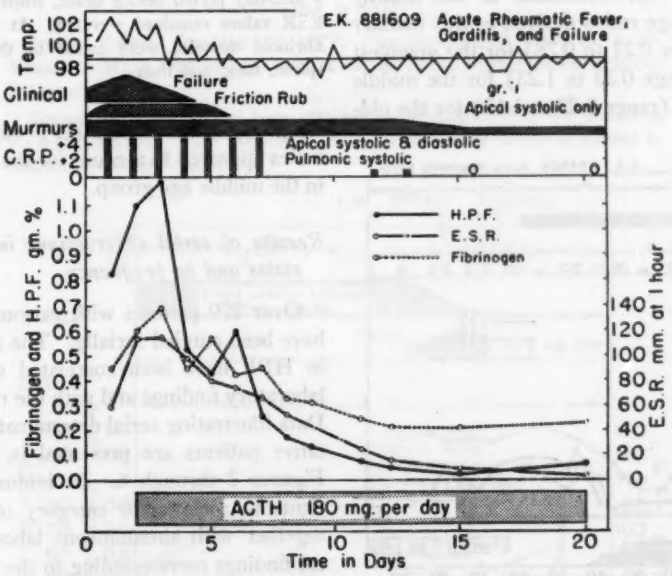

FIG. 2. CHART DEPICTING SERIAL CHANGES IN THE CLINICAL COURSE AND LABORATORY FEATURES OF SEVERE ACUTE RHEUMATIC FEVER WITH CARDITIS AND FAILURE IN A 12-YEAR-OLD GIRL

The clinical findings are presented schematically at the top of the chart, the C-reactive protein in the center, and the HPF (solid line), fibrinogen (dotted line) and ESR (dot-dash line) are given below. HPF reached 1.38 gm. per cent initially and fell abruptly to low levels with ACTH treatment. Fibrinogen and ESR, although elevated initially, fell more gradually to normal levels. Note that a high initial HPF/fibrinogen ratio decreased abruptly during recovery to the low values characteristic of normal individuals.

apparent difference in the mean values of children and adults, however, may be due to differences other than those determined by age alone, since the groups were composed of individuals of entirely different economic, social, and nutritional status.

Since it was evident early in this investigation that HPF resembled fibrinogen in certain respects, this protein was assayed simultaneously with HPF. The mean value for fibrinogen in 506 healthy individuals, given in section B of Table I, was approximately 0.22 gm. per cent, the oldest group of males having slightly higher values than the other groups. This mean fibrinogen value is somewhat lower than some normal data (9), but comparable to those of Saifer and Newhouse (5). Since the data (see below) on ill patients appeared to lend significance to changes in the ratio HPF/fibrinogen, this value was calculated for each pair of determinations in the healthy groups; the average ratios derived in this manner were 0.458 (range 0.21 to 0.78) for the youngest group, 0.709 (range 0.33 to 1.23) for the middle group, and 0.568 (range 0.27 to 1.17) for the oldest group. The higher HPF values found in females appeared to account for the high ratio values in the middle age group.

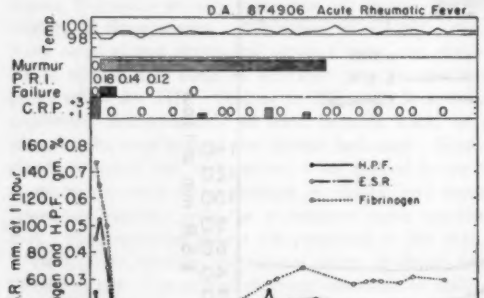

FIG. 3. CHART REPRESENTING SERIAL CLINICAL AND LABORATORY CHANGES IN AN 8-YEAR-OLD BOY WITH SEVERE RHEUMATIC FEVER WHO HAD BEEN PARTIALLY TREATED WITH ACTH PRIOR TO ADMISSION

Note that HPF became elevated during period of laboratory "rebound" after ACTH withdrawal, accompanied by slight fever, appearance of C-reactive protein in the serum and increase in sedimentation rate. Fibrinogen level elevation preceded the HPF rise by about 5 days. Return of these values to normal followed salicylate therapy.

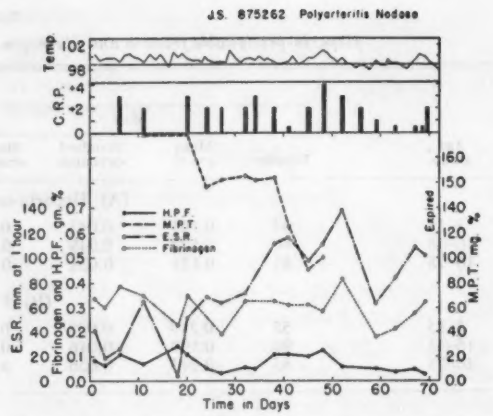

FIG. 4. J. S. SERIAL CHANGES IN HPF, FIBRINOGEN, AND ESR CORRELATED WITH CRP AND CLINICAL FINDINGS IN A 9-YEAR-OLD BOY WITH FULMINATING POLYARTERITIS

Note that HPF fell progressively to low levels in the 4 to 5-day period before death, while the fibrinogen and ESR values remained elevated. At autopsy widespread fibrinoid deposits were found in the kidney, adrenal, spleen, lung, and liver.

Results of serial observations in various disease states and in pregnancy

Over 200 patients with various types of illness have been studied serially. The observed changes in HPF have been correlated with clinical and laboratory findings and with the results of therapy. Data illustrating serial determinations in representative patients are presented in graphic form in Figures 2 through 6. Individual values for patients in each major category of illness studied, together with simultaneous laboratory and clinical findings corresponding to the HPF values, are given in Tables II through V. Where serial observations are available, the value given in the tables represents the initial HPF value in the series. Since the patient was usually acutely ill when first studied, this value usually represents the highest measured during the series.

Acute rheumatic fever. Data on forty children with acute rheumatic fever studied serially during the course of their disease are presented in Table II. Figures 2 and 3 illustrate the serial changes

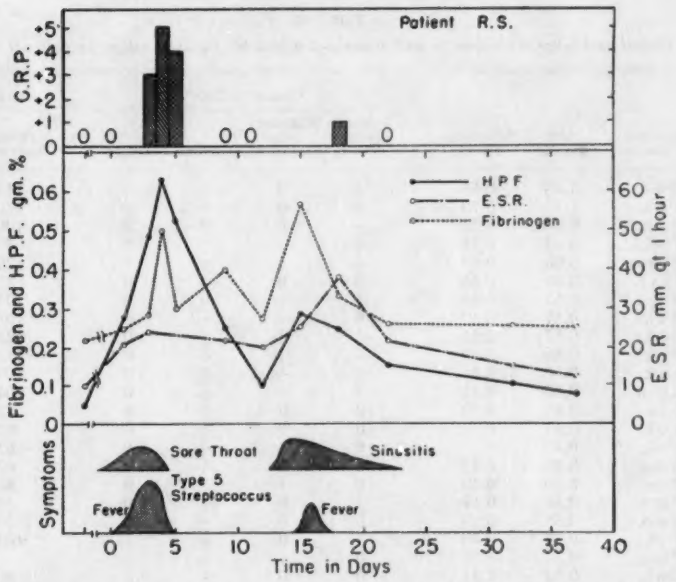

FIG. 5. SERIAL CHANGES IN HPF, FIBRINOGEN, AND ESR IN A 30-YEAR-OLD MALE DURING THE COURSE OF GROUP A, TYPE 5, STREPTOCOCCAL PHARYNGITIS

Normal HPF values had been obtained periodically during the preceding six-month period. Note rapidity of HPF elevation and return to normal in relation to clinical course.

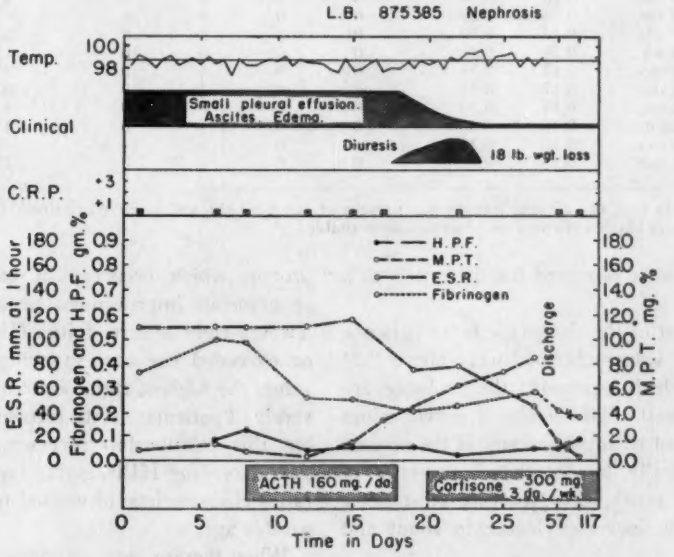

FIG. 6. SERIAL OBSERVATIONS OF HPF, FIBRINOGEN, ESR, AND MUCOPROTEINS (M.P.T.) DURING AN ACTH-INDUCED DIURESIS IN AN 8-YEAR-OLD BOY WITH NEPHROSIS

Note that despite changes in fibrinogen, ESR, and mucoprotein during the diuresis, the HPF levels remained relatively stationary and low.

TABLE II

Relation between clinical and laboratory activity and heparin-precipitable fraction values in patients with rheumatic fever

Patient	Age	Duration of disease	HPF gm. %	Fibrinogen gm. %	Clinical activity*				Laboratory activity		
					Temperature over 100° F	Migratory polyarthritis	Carditis	Cardiac failure	ESR mm./hour	CRP	Mucoprotein mg. tyrosine per 100 ml.
1	12	2 wk.	1.38	0.68	+	0	+	+	114	4+	18.10
2	8	1 wk.	1.10	0.70	+	0	+	+	120	4+	
3	11	2 wk.	0.67	0.52	+	0	0	0	100	4+	8.80
4		1 wk.	0.67	0.51	+	+	+	0	75	4+	15.10
5	14	2 days	0.66	0.63	+	+	+	+	129	4+	
6	77	1½ yr.	0.66	0.46	0	0	+	0	49	3+	2.20
7	16	1 mo.	0.65	0.46	+	+	0	0	93	2+	13.50
8	14	3 wk.	0.57	0.59	0	+	+	0	108	4+	14.70
9	7	2 wk.	0.57	0.64	+	+	+	0	121	4+	
10	7	3 wk.	0.56	0.49	+	+	+	0	114	3+	11.60
11	9	1 wk.	0.56	0.76	+	+	+	0	109	4+	13.80
12	12	2 mo.	0.50	0.53	+	+	+	0	104		
13	4	4 mo.	0.46	0.50	0	0	+	+	33	4+	
14	5	1 wk.	0.43	0.37	0	0	+	+	92	3+	
15	30	2 wk.	0.42		0	+	+	0	65	0	7.30
16	24	5 mo.	0.40	0.42	0	+	+	0	62	2+	6.30
17	13	8 mo.	0.40	0.40	0	+	+	0	46	1+	5.80
18	15	3 mo.	0.38	0.17	+	0	+	0	54	3+	7.80
19	10	1 mo.	0.38	0.19	0	0	+	0	23	1+	5.10
20	14	1 wk.	0.36	0.46	+	0	+	+	103	2+	9.30
21	9	2 mo.	0.34		0	+	+	+			8.80
22	11	3 wk.	0.32	0.22	0	0	+	+	80	2+	7.50
23	7	1 mo.	0.31	0.45	+	+	+	+	39	2+	10.65
24	4	3 wk.	0.30	0.38	0	+	+		122		9.35
25	7	2 wk.	0.30	0.29	+	+	+	+	49	2+	8.20
26	11	2 wk.	0.27	0.28	0	0	+	0	18		2.58
27	12	3 wk.	0.27	0.29	0	0	+	0	47	0	8.00
28	13	8 mo.	0.27	0.51	0	0	+	0	103	1+	6.68
29	4	3 mo.	0.26	0.47	0	+	+	0	56	4+	8.20
30	12	3 mo.	0.26	0.37	0	0	+	0	36	0	
31	9	3 mo.	0.26	0.74	0	+	+	0	93	4+	16.00
32	12	3 mo.	0.24	0.34	0	0	0	0	6	0	7.10
33	38	4 wk.	0.23	0.19	0	+	+	0	22	1+	7.90
34	18	1 wk.	0.20	0.25	0	+	0	0	15	0	6.08
35	12	1 mo.	0.17	0.38	0	0	+	0	89	0	4.10
36	5	1 mo.	0.17	0.42	+	0	+	0	62	2+	10.70
37	7	1 mo.	0.17	0.20	0	0	0	0	43	0	7.80
38	11	2½ mo.	0.16	0.28	0	0	+	0	44	0	4.60
39	13	3 mo.	0.16	0.21	+	0	+	+	9	2+	3.40
40	7	1 mo.	0.15	0.22	0	0	+	0	15	0	6.50

* "+" indicates that the clinical feature was present at the time the values were obtained; 0 indicates that these features were absent; blank indicates no observation available.

in HPF which were observed for the group as a whole.

In all but eight of the rheumatic fever patients, the initial HPF values obtained were above 0.24 gm. per cent, which represents the probable upper limit of normal. The height of initial values roughly correlated with the severity of the process as gauged clinically by "toxicity," presence of pericardial friction rub, fever, joint manifestations, and by markedly increased leucocyte count and ESR.

The patient whose clinical course is shown in Figure 2 illustrates the reversal of the relationship between the absolute values for HPF and clottable protein which occurred in most patients with spontaneous improvement or successful therapy. Twenty-eight of forty initial HPF values equalled or exceeded the corresponding clottable protein value, the highest ratio occurring in the most severely ill patients. With recovery both values fell, but this relationship reversed, clottable protein then exceeding HPF, giving low HPF/fibrinogen ratios characteristic of normal individuals of comparable age.

When therapy was withdrawn abruptly, resulting in the so-called "rebound" phenomenon, HPF values again rose above normal. An example of this type of change is illustrated in Figure 3.

"Collagen" diseases. The hypothesis that the heparin-precipitable component might represent a fibrinoid "precursor" (1, 2) led to serial examination in patients with diseases in which fibrinoid deposition represents significant histopathological change. Table III summarizes the representative values obtained in patients with juvenile and adult forms of rheumatoid arthritis, acute lupus erythematosus disseminatus, scleroderma, dermatomyositis, polyarteritis, and anaphylactoid purpura. Patients with rheumatoid arthritis, like the rheumatic fever group, had high HPF values when their disease was most severe, with high fever, joint involvement, leucocytosis, and the characteristic skin rash. When alleviation of these manifestations was brought about by cortisone or salicylate administration, or when spontaneous remissions occurred, the values returned to normal or low levels.

On the other hand, the patients with disseminated lupus erythematosus and polyarteritis were found to have HPF values in the normal range for age, or even below normal levels, in spite of high fever, severe illness, elevated clottable protein values, strongly positive CRP, extremely high ESR and mucoprotein values. In one patient having polyarteritis (Figure 4) HPF values fell to extremely low levels while clottable protein values remained relatively unchanged during the week prior to demise. At autopsy widespread vascular lesions with prominent deposition of fibrinoid were found.

Infectious diseases. High HPF levels were found in patients with various acute bacterial infections, as shown in Table IV.

Marked initial elevation of HPF values was observed in patients having meningitis due to *H. influenza, Str. pneumoniae,* or *Niesseria intracellularis,* and in those having streptococcal pharyngitis (Figure 5), urinary tract infection, bacterial endocarditis, and cryptococcosis. These high initial HPF levels returned to normal with effective antibiotic therapy. Again, the reversal in the relationship of HPF and clottable protein as recovery ensued was found.

In contrast to the elevation of HPF observed in acute bacterial infections, patients with common viral infections studied were found to have normal or only slightly elevated HPF values.

Neoplastic diseases. Nineteen patients of various ages with neoplastic disease of various types were studied. The values obtained are summarized in Table V. High values were found in patients whose lesions were extensive or widely dis-

TABLE III

Heparin-precipitable fraction in patients with various "collagen" disorders

Patient	Age	Disease and activity	HPF gm. %	Fibrinogen gm. %	ESR mm./hr.	CRP
1	5	Severe juvenile rheumatoid arthritis	0.73	0.66	91	3+
2	9	Severe juvenile rheumatoid arthritis	0.52	0.39	84	4+
3	4	Severe juvenile rheumatoid arthritis	0.47	0.43	104	2+
4	1	Severe juvenile rheumatoid arthritis	0.47	0.36	130	4+
5	50	Severe chronic rheumatoid arthritis	0.44	0.71	113	4+
6	22	Severe rheumatoid arthritis—steroid treatment	0.38	0.42	104	3+
7	6	Severe juvenile rheumatoid arthritis—salicylate treatment	0.33	0.64	54	4+
8	15	Acute disseminated lupus erythematosus	0.32	0.64	142	4+
9	12	Acute disseminated lupus erythematosus	0.16	0.51	123	4+
10	21	Acute disseminated lupus erythematosus	0.22	0.21	3	0
11	32	Acute disseminated lupus erythematosus—cortisone treatment	0.08	0.15		2+
12	70	Acute disseminated lupus erythematosus—cortisone treatment	0.08	0.32	103	2+
13	9	Severe dermatomyositis—cortisone treatment	0.21	0.20	9	0
14	5	Schonlein-Henoch purpura	0.35	0.25	54	2+
15	4	Dermatomyositis	0.54	0.58	85	1+
16	12	Scleroderma	0.22	0.25	49	1+
17	9	Fulminating polyarteritis	0.04	0.32	103	2+
18	28	Fulminating polyarteritis	0.18	0.43	63	3+

TABLE IV

Heparin-precipitable fraction values in patients with various infections

Patient	Age	Type of infection	Micro-organism	Duration	HPF gm. %	Fibrinogen gm. %	ESR mm./hr.	C-reactive protein
1	3 yr.	Meningitis-septicemia	*H. influenza*	1 day	1.08	0.88	104	4+
2	22 mo.	Meningitis-septicemia	*H. influenza*	4 days	0.77	0.53	123	4+
3	16 mo.	Meningitis-septicemia	*H. influenza*	10 days	0.36	0.48	108	3+
4	1 yr.	Meningitis-septicemia	*H. influenza*	6 days	0.31	0.35	33	3+
5	1 yr.	Meningitis-septicemia	*H. influenza*	6 days	0.31	0.38	81	1+
6	6½ yr.	Meningitis	*N. intracellularis*	2 days	0.83	0.36	40	3+
7	10 yr.	Meningitis-septicemia	*N. intracellularis*	3 days	0.69	0.54	53	3+
8	3 yr.	Meningitis-septicemia	*N. intracellularis*	1 wk.	0.38	0.51	109	3+
9	3 yr.	Meningitis-septicemia	*N. intracellularis*	1 day	0.33	0.78		4+
10	14 yr.	Meningitis	*D. pneumoniae*	1 wk.	0.40	0.53	71	4+
11	10 yr.	Meningitis (partially treated)	*D. pneumoniae*	1 wk.	0.23	0.47	106	0
12	8 yr.	Pharyngitis	*Str. hemolyticus*, Group A	6 days	0.68	0.37	51	3+
13	30 yr.	Pharyngitis	*Str. hemolyticus*, Type 5	4 days	0.65	0.50	22	4+
14	16 yr.	Scarlet fever	*Str. hemolyticus*, Type 18	5 days	0.57	0.31		
15	1½ yr.	Pharyngitis	*Str. hemolyticus*, Group A	3 days	0.41	0.42	34	0
16	7 yr.	Mastoiditis, chronic	Various	2 yr.	0.22	0.35	98	0
17	13 yr.	Bronchiectasis, chronic	Various	10 yr.	0.29	0.35	13	0
18	4 yr.	Acute and chronic urinary tract infection	*E. coli*	3 days	0.87	0.58	106	4+
19	4 yr.	Acute urinary tract infection	*E. coli*	1 day	0.30		30	4+
20	14 yr.	Subacute bacterial endocarditis	*Str. viridans*	2 mo.	0.60	0.42	110	3+
21	14 yr.	Acute appendicitis		1 day	0.27	0.40	57	3+
22	4 yr.	Generalized infection	*Cryptococcus neoformans*	Unknown	0.60	0.54	64	4+
23	9 yr.	Polycythemia due to cong. H. D.; acute pulmonary infection	*Histoplasma capsulatum*	3 wk.	0.21	0.14	0	4+
24	6 yr.	Measles		44 days	0.32	0.38	42	
25	5 yr.	Measles		6 days	0.16	0.29	38	1+
26	3 yr.	Common cold		2 days	0.15	0.24		
27	6 yr.	Encephalitis	Mumps	4 days	0.18	0.20		

TABLE V

Heparin-precipitable fraction values in patients with various neoplastic diseases

Patient	Age	Type of tumor	HPF gm. %	Fibrinogen gm. %	ESR mm./hr.	CRP
1	7 yr.	Hodgkin's disease	0.83	0.67	74	4+
2	7 mo.	Cavernous hemangioma	0.66	0.35	49	
3	7 yr.	Hodgkin's disease	0.61	0.52	106	3+
4	54 yr.	Metastatic carcinoma of the lung	0.55	0.58	29	3+
5	68 yr.	Metastatic carcinoma of the lung	0.37	0.30	22	
6	10 yr.	Acute leukemia	0.31	0.32	78	
7	9 yr.	Acute leukemia	0.29	0.37	40	1+
8	4 yr.	Metastatic Wilm's tumor	0.23	0.25	26	
9	9 yr.	Jaw sarcoma	0.22	0.12	23	0
10	13 yr.	Myelogenous leukemia	0.19	0.27	22	+
11	7 yr.	Disseminated lymphoma	0.18	0.28	40	4+
12	8 mo.	Neuroblastoma	0.13	0.32	42	
13	6 mo.	Wilm's tumor	0.13	0.32	42	0

seminated, and in whom constitutional manifestations were severe.

Nephrosis. Sixteen of the 21 nephrotic patients studied had normal or low HPF levels, although clottable protein values were frequently very high and CRP was present in the serum. Serial studies during the course of steroid-induced diureses in these patients (Figure 6) failed to demonstrate any significant changes in HPF in spite of abrupt reduction of ESR, prompt elevation of mucoprotein, and reduction of clottable protein to within normal limits. This mucoprotein change has been observed previously (10).

Pregnancy. Elevated HPF values were found as early as the second month of the first trimester of pregnancy; the levels gradually rose during pregnancy to a maximal value at delivery, and fell to normal about two weeks post partum. Patients

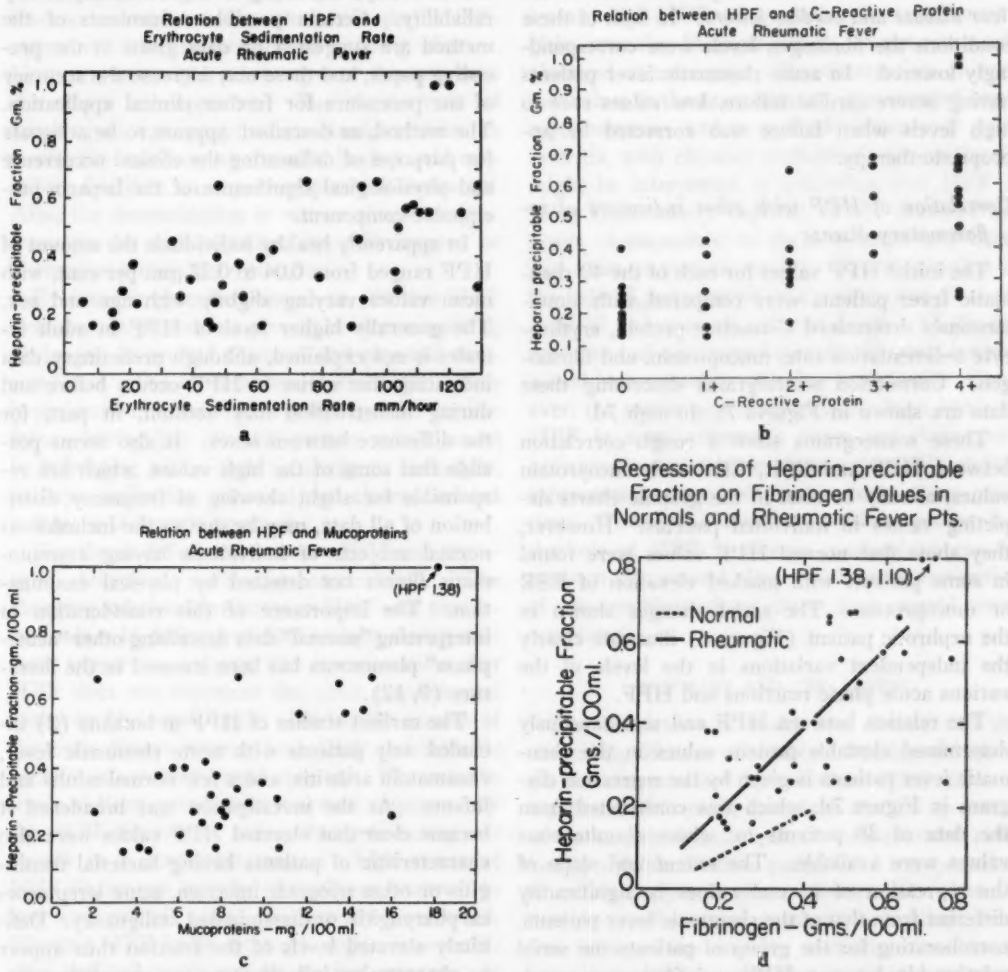

FIG. 7 a–d. CORRELATION SCATTERGRAMS RELATING HPF VALUES TO SIMULTANEOUSLY DETERMINED ESR, C-REACTIVE PROTEIN, MUCOPROTEIN, AND FIBRINOGEN VALUES IN 40 PATIENTS HAVING ACUTE RHEUMATIC FEVER

Note that a general correlation of the values is shown, but that considerable scatter is evident. In Figure 7d the black line gives the regression of HPF on fibrinogen for the points shown. This should be compared with the similar regression coefficient for 122 normals of similar age represented by the dotted line (points not shown). The slope of the regression lines, as well as the range of values, differs significantly in the two groups.

with manifestations of pre-eclampsia had higher values than normal women at the same stage of pregnancy. Newborn infants, in contrast to their mothers at term, were found to have very low values, ranging from no measurable level to 0.06 gm. per cent. During the first 72 hours of life, however, these values rose to within the normal range for older infants. These data will be presented in detail elsewhere (11).

Low values were found in patients with severe *liver disease* and *cardiac failure*. In both of these conditions the fibrinogen levels were correspondingly lowered. In acute rheumatic fever patients having severe cardiac failure, low values rose to high levels when failure was corrected by appropriate therapy.

Correlation of HPF with other indicators of inflammatory disease

The initial HPF values for each of the 40 rheumatic fever patients were compared with simultaneously determined C-reactive protein, erythrocyte sedimentation rate, mucoprotein, and fibrinogen. Correlation scattergrams describing these data are shown in Figures 7a through 7d.

These scattergrams show a rough correlation between HPF and ESR, CRP and mucoprotein values, as may be seen in the graphic charts depicting values in individual patients. However, they show that normal HPF values were found in some patients with marked elevation of ESR or mucoprotein. The serial changes shown in the nephrotic patient (Figure 6) illustrate clearly the independent variations in the levels of the various acute phase reactions and HPF.

The relation between HPF and simultaneously determined clottable protein values in the rheumatic fever patients is given by the regression diagram in Figure 7d, which was constructed from the data of 38 patients on whom simultaneous values were available. The extent and slope of the regression of normal values is significantly different from that of the rheumatic fever patients, corroborating for the group of patients the serial relationship between HPF and fibrinogen previously shown for individual patients.

DISCUSSION

The data presented in this paper allow a quantitative analysis of the occurrence of a heparin-precipitable, cold-insoluble plasma component in normal individuals and patients with various acute diseases. The method employed routinely for estimating the amount of this component in plasma has proven to be relatively simple and reliable, giving results which are reproducible in replicate samples within ± 0.02 gm. per cent. Serial daily determinations in individuals with various diseases, and during health, gave data which plotted out smoothly, indicating a degree of day-to-day reliability. Certain possible refinements of the method are suggested by data given in the preceding paper, and these may increase the accuracy of the procedure for further clinical application. The method, as described, appears to be adequate for purposes of delineating the clinical occurrence and physiological significance of the heparin-precipitable component.

In apparently healthy individuals the amount of HPF ranged from 0.04 to 0.27 gm. per cent, with mean values varying slightly with age and sex. The generally higher level of HPF in adult females is not explained, although preliminary data indicating that a rise in HPF occurs before and during menstruation may account, in part, for the difference between sexes. It also seems possible that some of the high values, which are responsible for slight skewing of frequency distribution of all data, may be due to the inclusion as normal subjects of individuals having asymptomatic illness not detected by physical examination. The importance of this consideration in interpreting "normal" data describing other "acute-phase" phenomena has been stressed in the literature (9, 12).

The earliest studies of HPF in humans (2) included only patients with acute rheumatic fever, rheumatoid arthritis, and a few normal adults and infants. As the investigation was broadened it became clear that elevated HPF values were also characteristic of patients having bacterial meningitis or other pyogenic infection, acute streptococcal pharyngitis, or disseminated malignancy. Definitely elevated levels of the fraction thus appear to characterize inflammatory or necrotizing disorders in general rather than any specific disease process. Furthermore, data correlating HPF with the clinical status of the patient indicate that the levels in the plasma are roughly proportional to the severity of the disease under study. There-

fore, the clinical studies indicate that the occurrence of high levels of a cold-insoluble, heparin-precipitable component in plasma may be added to the long list of so-called "acute-phase" reactions (12) occurring in the circulating blood of acutely ill patients.

The question arises as to the possible practical application of a test for this component of plasma in clinical practice. This study suggests that determination of HPF may provide information at least as valuable as the other non-specific indicators of inflammatory or necrotizing disease. However, no unique advantages of this test over the others are apparent. The requirement of handling at low temperatures would restrict its availability to those laboratories having cold centrifuge facilities, an important practical limitation. Also, the determination is not as simple to perform as the sedimentation rate, fibrinogen, or C-reactive protein determination.

Evidence is presented elsewhere (1) that HPF from human plasma is a protein closely resembling fibrinogen in several chemical and physical characteristics. The observations of low HPF values in patients with liver disease and congestive cardiac failure having low clottable protein values would be in keeping with a close similarity to fibrinogen, perhaps indicating identical or closely related pathways of hepatic synthesis.

Significant differences between HPF and fibrinogen, however, were also encountered. The changing relationships between HPF and fibrinogen values in patients studied serially during disease appear to support the interpretation that HPF does not represent the same physiological entity as fibrinogen itself. During the course of the acute disease process, both HPF and clottable protein values were found elevated. The relative levels of these parameters, however, expressed by the HPF/fibrinogen ratio, changed during recovery in a consistent manner; high ratios of HPF/fibrinogen in very acute illness returned to low and normal ratios as recovery progressed. Elevated HPF levels appeared earlier in acute illness and fell more rapidly during recovery, in general, than the clottable fraction levels. An alternative interpretation of the high HPF/fibrinogen ratios during acute illness would be that they reflect marked decrease in the clottability of fibrinogen rather than a relative increase in HPF. Such a hypothesis is neither supported nor excluded by these data, but deserves further investigation.

The possibility that HPF may, like fibrinogen, play a role in the pathogenesis of fibrinoid lesions has been suggested elsewhere on the basis of observations in experimental animals (13). The question as to its participation in the deposition of fibrinoid in human lesions remains a speculative matter. In some of the conditions characterized by very high HPF values, such as bacterial meningitis, extensive fibrinoid lesions are not commonly observed at necropsy. The observation of very low values in the cases of acute disseminated lupus erythematosus, and in a single instance of polyarteritis, with elevated clottable fibrinogen levels, might be interpreted as indicating that HPF is being continuously depleted as the fibrinoid deposits characteristic of these diseases are being laid down in various organs. The observation in the case of polyarteritis of a progressive decrease in HPF to low levels prior to death, with the development of widespread vascular fibrinoid lesions, would be compatible with this hypothesis. However, the occurrence of abnormally high levels of HPF in acute rheumatic fever and rheumatoid arthritis favors the interpretation that HPF simply represents another inexplicable acute phase reaction. Therefore, no interpretations concerning the role of HPF in disease mechanisms can be made at the present time. Further investigation of the problem along these lines would seem warranted.

SUMMARY AND CONCLUSION

The occurrence in health and disease and the clinical significance of a protein fraction of human plasma characterized by cold-insolubility in combination with heparin have been studied by means of an empirical technique of measurement sufficiently reliable to allow quantitative interpretation of serial changes in plasma levels. The data allow the conclusions that normal individuals have low levels of the fraction, and that these levels become greatly increased as a result of most acute inflammatory or necrotizing diseases. The appearance of the fraction in increased amounts represents another reaction in the category of "acute-phase" phenomena. The data support the other items of evidence indicating that the fraction may

fulfill a different physiological role from clottable protein. The nature of this function is as yet unrevealed.

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THE MECHANISM OF GLUTATHIONE DESTRUCTION AND PROTECTION IN DRUG-SENSITIVE AND NON-SENSITIVE ERYTHROCYTES. *IN VITRO* STUDIES

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When the anti-malarial drug primaquine or certain related aniline derivatives, such as sulfanilamide and acetanilid, are administered to sensitive individuals, an acute hemolytic anemia develops (1, 2). Such sensitivity is very rare among Caucasians but occurs in approximately 10 per cent of American Negroes (3). Investigation of this phenomenon by means of red cell survival studies established that sensitivity to the hemolytic action of primaquine was due to an intrinsic abnormality of the red blood cells of sensitive subjects (4). Extensive studies of these erythrocytes demonstrated, however, that sensitivity to these drugs is not merely another manifestation of a previously described red cell abnormality. It was shown, for example, that the defect of drug-sensitive cells is not associated with any alteration in fragility to chemical, osmotic and mechanical lysis, electrophoretic mobility of the hemoglobin, susceptibility to alkali denaturation, antigenic pattern, or Coomb's test (5).

When the drug was administered continually to a sensitive individual, hemolysis stopped after the initial hemolytic crisis and the blood counts returned spontaneously to normal (6). By labeling red cells with a limited age span with Fe^{59} we were able to explain this phenomenon, demonstrating that only the older members of the red cell population were destroyed through the action of drug (7). This finding led us to suggest that primaquine sensitivity might be due to a deficiency in an enzyme system that decreased in activity as the red cell aged, a condition which is known to be met by a number of red cell enzymes (8).

We therefore examined various enzyme systems in red cells, which for one reason or another might be implicated in drug sensitivity. These studies indicated that sensitivity to these drugs was not due to a defect in catalase, carbonic anhydrase, or cholinesterase activity. They also demonstrated that glycolysis was sufficiently normal to result in normal incorporation of radioactive phosphorus and that blocking glycolysis with sodium fluoride did not cause non-sensitive cells to behave like sensitive cells in the *in vitro* study system we had developed (8). Measurement of the reduced glutathione (GSH) content of drug-sensitive cells revealed that they were deficient in this compound (8). When the GSH content of sensitive cells was measured during drug administration it was found that an abrupt fall in the average red cell GSH concentration preceded the major portion of the hemolytic episode, but that as hemolysis progressed, the average red cell GSH returned to pre-treatment levels (9). This suggested that GSH was destroyed in the older, sensitive red cells and that subsequently the GSH deficient cells were removed from the circulation. Whether the fall in GSH level is the direct cause of the *in vivo* destruction of the red cell or merely one manifestation of a large number of incidental drug-induced alterations in the chemistry of the cell is a question that still requires an answer. However, it seemed reasonable that GSH might play an important role in drug-induced hemolyis, and that therefore further study of the destruction of red cell glutathione was warranted.

MATERIALS AND METHODS

Blood donors. Most individuals designated as "sensitive" or "non-sensitive" had been given 30 mg. of primaquine daily to establish their reaction to the drug at some time prior to the studies reported in this paper. None had received drug for at least several weeks and some had not been exposed to it for several years.[1] In

[1] The authors are indebted to Drs. Alf S. Alving, C. Larkin Flanagan, and Paul Carson for making most of these blood samples available for study.

some instances it was necessary to use red cells from a donor whose sensitive status was established by *in vitro* testing, using the two methods that we have described (10, 11). In experiments using these cells, they are referred to as "sensitive (by test)." Because of the negligible incidence of drug sensitivity among Caucasians (3) samples from Caucasian donors were regarded as being non-sensitive and are referred to as being "non-sensitive (Caucasian)."

GSH stability test. The method of testing the stability of red cell glutathione has been presented in detail elsewhere (11). The GSH content of the blood sample was measured, and 1-ml. aliquots of the well-oxygenated sample were added to test tubes containing 5 mg. of acetylphenylhydrazine. After 2 hours' incubation at 37° C, GSH determinations were carried out on the incubated aliquots.

Glutathione determinations. Reduced glutathione (GSH) determinations were carried out by our modification (11) of the method of Grunert and Phillips (12). Total glutathione (GSH plus oxidized glutathione (GSSG)) was determined by electrolytically reducing a 10-ml. aliquot of metaphosphoric acid filtrate obtained by this method. Electrolytic reduction was carried out over mercury in a 4.6 cm.-diameter beaker for 30 minutes at 40 ma. according to the method of Dohan and Woodward (13).

Hemoglobin solutions. Destromatized hemoglobin solutions were prepared by a modification of the method of Drabkin (14). Red cells washed three times with 0.9 per cent sodium chloride solution were shaken with 1 volume of water and 0.4 volumes of toluene for one-half hour and then centrifuged. The hemoglobin layer was filtered. The hemoglobin solution was then dialyzed in 18/32 inch Visking cellophane casing against large volumes of distilled water at 4° C for 72 hours. Erythrocytes from a non-sensitive (Caucasian) donor were used.

a. Methemoglobin was prepared by suspending the red cells in 1 per cent sodium nitrite for one-half hour before washing with saline.
b. Cyanmethemoglobin was prepared by adding 0.1 per cent sodium cyanide to the red cells suspended in 1 per cent sodium nitrite before washing with saline.
c. Carboxyhemoglobin was formed by passing carbon monoxide through the dialyzed, destromatized hemoglobin solution.
d. "Acetylphenylhydrazine-modified hemoglobin" was formed by incubating 1-ml. aliquots of blood with 5 mg. acetylphenylhydrazine before washing with sodium chloride solutions. Dialysis was carried out for 48 hours. Although it seemed reasonable that this procedure would remove unbound acetylphenylhydrazine from the solution, no direct tests for retained acetylphenylhydrazine have been carried out. Measurements of absorption spectra were carried out in the Beckman Direct Transmittance Recording Spectrophotometer, Model DK, in the range 325 to 800 mμ.

Substrates and coenzymes. Reduced glutathione, the barium salts of glucose-6-phosphate, fructose-6-phosphate and ribose-5-phosphate, inosine, adenosine triphosphate (ATP), ribose, and fructose diphosphate were obtained from the Nutritional Biochemicals Corporation. The barium salt of 6-phosphogluconic acid and diphosphopyridine nucleotide (DPN) were obtained from Delta Chemical Works. Methylene blue was obtained from the National Aniline Division, Allied Chemical & Dye Corporation. Sodium fumarate was prepared by neutralizing fumaric acid, recrystallized from the practical grade, Eastman Kodak, with sodium hydroxide to a pH of 7.0. Sodium pyruvate and sodium malate were prepared in the same fashion from pyruvic acid, kindly supplied by Dr. E. S. G. Barron, and from malic acid, obtained from the Pfanstiel Chemical Company. Sodium lactate was prepared by heating lactic acid, Baker Chemical Company, with an excess of sodium hydroxide at 80° C for 20 minutes, then adjusting the pH to 7.0 with hydrochloric acid. Nicotinamide, U.S.P., was obtained from the New York Quinine Chemical Company. Reduced triphosphopyridine nucleotide (TPNH) and reduced DPN (DPNH) were obtained from Sigma Chemical Company.

Sodium salts were prepared from barium salts immediately prior to use. One and one-half ml. of distilled water

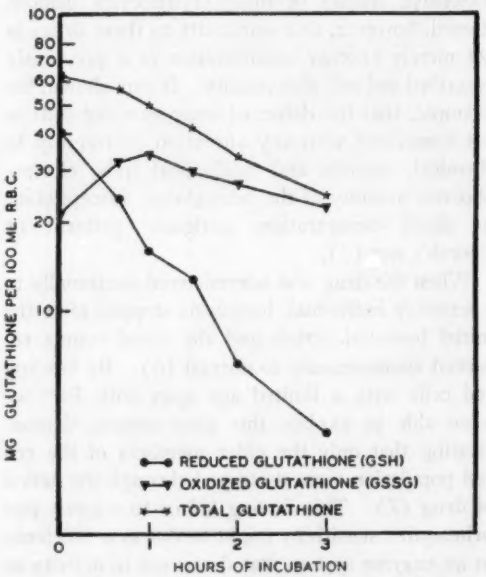

FIG. 1. THE FATE OF THE GSH OF DRUG-SENSITIVE RED CELLS INCUBATED WITH ACETYLPHENYLHYDRAZINE

One-ml. aliquots of blood from a sensitive (by test) subject were incubated with 5 mg. acetylphenylhydrazine. As the GSH concentration fell there was a rise in the GSSG concentration, followed after 1 hour by a decline. A gradual decrease in total glutathione concentration took place throughout the 3-hour experiment.

and 0.1 ml. of 1 N hydrochloric acid were added to 64 μM, by weight, of the barium salt as supplied by the manufacturer. After solution of the barium salt 0.3 ml. of 0.213 M sodium sulfate was added, and the barium sulfate precipitate removed by centrifugation. The supernatant was neutralized with 0.1 ml. of 1 N sodium hydroxide. The other substrates and the coenzymes were dissolved directly in water. Fructose diphosphate was not entirely soluble in 0.032 M concentration and was used as a suspension. The materials were assumed to be entirely pure as supplied by the manufacturer, and the yield in preparation was assumed to be 100 per cent, except in the case of DPNH and TPNH, the concentrations of which were measured spectrophotometrically.

EXPERIMENTAL AND RESULTS

The glutathione instability of sensitive red cells

One-ml. aliquots of blood from 5 sensitive and 7 non-sensitive individuals have been incubated with 5 mg. acetylphenylhydrazine for 2 hours. The GSH content of the red cells of non-sensitive individuals changed very little while that of sensitive individuals fell markedly (11). The subsequent experiments were designed to clarify the mechanism of the destruction of the GSH and the mechanism of the difference in the response of drug-sensitive and drug-non-sensitive cells to incubation with acetylphenylhydrazine.

The fate of the GSH of sensitive red cells incubated with acetylphenylhydrazine

One-ml. aliquots of blood from a sensitive (by test) subject were incubated with 5 mg. acetylphenylhydrazine using the "glutathione stability test" technique. The reduced (GSH) and oxidized (GSSG) glutathione contents of the red cells were determined initially and after incubation for 40, 60, 90, 120, and 180 minutes. The results of this experiment are presented in Figure 1. The GSH concentration of the erythrocytes fell rapidly, the characteristic response of sensitive red cells to incubation with acetylphenylhydrazine. The GSSG, however, showed an initial rise, the total glutathione (GSH and GSSG) concentration falling only slightly, indicating that most of the "destroyed" GSH had been oxidized to GSSG. After 1 hour, the GSSG concentration also began to decline. Thus, there was a gradual decline in total glutathione throughout the 3-hour experiment.

The mechanism of the destruction of GSH

The degree of oxygenation of the sensitive red cell influences markedly the destruction of its GSH content by acetylphenylhydrazine. Oxygen, carbon dioxide, or carbon monoxide have been passed through blood from a non-sensitive individual and GSH stability tests carried out. The stability of the GSH of non-sensitive cells was not affected appreciably by any of the gases used. In contrast, the response of the GSH of sensitive cells to incubation with acetylphenylhydrazine was influenced markedly. The oxygenated sensitive cells showed the characteristic fall in GSH level, but there was very little drop in GSH level in the sensitive cells that had been gassed with carbon dioxide and none when carbon monoxide was used (11). This suggested that hemoglobin might play an important role in mediating the reaction between acetylphenylhydrazine and GSH. Therefore, studies of the effect of hemoglobin on GSH solutions incubated with acetylphenylhydrazine were carried out.[2]

One ml. of a 307 mg. per cent (10 mM) GSH solution was added to tubes containing 9 ml. of 8 grams per cent, 2 grams per cent, and 0.5 grams per cent oxyhemoglobin, 8 grams per cent methemoglobin, 8 grams per cent cyanmethemoglobin, and 8 grams per cent carboxyhemoglobin, and a tube containing 9 ml. of water. The GSH content of 1-ml. aliquots of these mixtures was determined within a few minutes, and 1-ml. aliquots were incubated with and without 5 mg. acetylphenylhydrazine at 37° C for 2 hours after which time the GSH concentration was measured. The results of this experiment are presented in Table I, which demonstrates that the destruction of GSH by acetylphenylhydrazine does not take place when

[2] A complicating factor in this portion of the investigation was that some hemoglobin solutions, especially those which had been stored for more than one week at 4° C, demonstrated considerable GSH destructive properties. This destructive property is only very slightly influenced by formation of methemoglobin or cyanmethemoglobin from the original solution and therefore does not represent activity of traces of methemoglobin contaminating the oxyhemoglobin solution. By using hemoglobin solutions only several days old, it was possible to avoid, for the most part, the direct effect of hemoglobin solutions on GSH and thus to study the effect of acetylphenylhydrazine-induced destruction of GSH with a minimum of interference.

TABLE I

The effect of several hemoglobin compounds on GSH with and without acetylphenylhydrazine

Hemoglobin compound	Hemoglobin concentration* *grams per cent*	Mg. per cent GSH prior to incubation	Mg. per cent GSH after 2 hours incubation *without* acetylphenylhydrazine	Mg. per cent GSH after 2 hours incubation *with* 5 mgm. acetylphenylhydrazine
Control	0	30.7	31.5	30.9
Oxyhemoglobin	.45	30.1	—	29.7
Oxyhemoglobin	1.8	27.2	—	19.5
Oxyhemoglobin	7.2	25.1	20.3	8.8
Methemoglobin	7.2	26.6	16.9	14.9
Cyanmethemoglobin	7.2	30.3	28.7	25.4
Carboxyhemoglobin	7.2	24.7	22.9	24.5
Acetylphenylhydrazine-modified hemoglobin	6.2	25.8	7.4	—

* Measured as cyanmethemoglobin.

these two compounds are mixed in the pure form. Furthermore, the data in this table demonstrate that relatively large concentrations of oxyhemoglobin are necessary to mediate the reaction. Methemoglobin itself destroys only a moderate amount of GSH, and the presence of acetylphenylhydrazine in the system has little additional effect. Cyanmethemoglobin is relatively inert as is carboxyhemoglobin. It is apparent from the data presented that the recovery of GSH in the presence of large amounts of some of the hemoglobin compounds was appreciably less than 100 per cent. This unexplained finding has been quite consistent, although recovery of small amounts of GSH added to whole blood is complete (12, 15).

The effect of acetylphenylhydrazine-modified hemoglobin, prepared in such a way as to remove all or most of the acetylphenylhydrazine from the solution (see Materials and Methods), was also determined. One part of 307 mg. per cent GSH solution was added to 9 parts of 6.9 grams per cent acetylphenylhydrazine modified hemoglobin (measured as cyanmethemoglobin at 540 mμ). The recovery of GSH was rather poor, only 25.8 mg. per cent could be recovered immediately. After incubation for 2 hours at 37° C, only 7.4 mg. per cent could be measured (Table I). Thus, oxyhemoglobin modified by acetylphenylhydrazine possessed the same order of destructiveness toward GSH as did acetylphenylhydrazine in combination with 8 grams per cent oxyhemoglobin. Further studies indicate that the addition of carbon monoxide and of 98 mg. per cent sodium cyanide fail to modify its GSH-destructive effect. The absorption spectrum of the approximately 6.9 grams per cent acetylphenylhydrazine-modified hemoglobin solution diluted 1 to 100 in 0.005 M phosphate buffer at pH 7.0 has been measured in the original state, with addition of oxygen, carbon monoxide, sodium hydrosulfite, and sodium cyanide, final concentration 0.0004 M. The spectra of these preparations are illustrated in Figure 2 and the absorption maxima

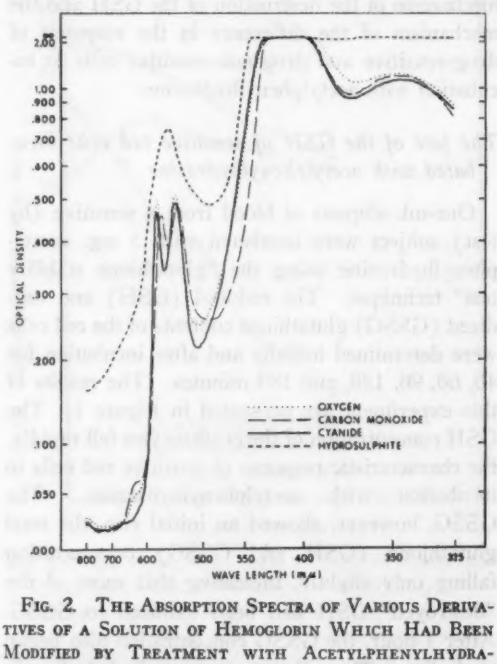

FIG. 2. THE ABSORPTION SPECTRA OF VARIOUS DERIVATIVES OF A SOLUTION OF HEMOGLOBIN WHICH HAD BEEN MODIFIED BY TREATMENT WITH ACETYLPHENYLHYDRAZINE (SEE METHODS)

The spectrum of the unmodified pigment very closely resembled that observed after treatment with oxygen.

TABLE II

Absorption maxima of "acetylphenylhydrazine modified hemoglobin"

(All measurements were carried out in .005 M phosphate buffer, pH 7.0, with the Beckman direct transmittance recording spectrophotometer, Model DK.)

Treatment	Absorption maxima mμ				
None	628–608*	579	543	425–393*	350
Oxygen	630–615*	580	543	428–393*	350
Hydrosulfite	—	554	—	428	—
Carbon monoxide	630–615*	571	539	430–395*	364–354*
Cyanide	—	577	543	432–397*	345

* Plateau, approximate range.

are presented in Table II. The spectra do not differ significantly from those of unaltered hemoglobin except for a small plateau in the 608 to 630 mμ region. This disappears on treatment with hydrosulfite or cyanide, and apparently represents a trace of methemoglobin.

The biochemistry of the protection of red cell GSH against the effect of acetylphenylhydrazine

Early in the course of our studies of the GSH stability test we discovered that if blood samples were allowed to stand at room temperature for 24 hours, non-sensitive red cells lost their capacity to protect their GSH against acetylphenylhydrazine, but that their protective mechanism remained intact if an excess of glucose was added to the blood (11).

Further studies of the dependence of the GSH protecting mechanism on glucose have been carried out. Plasma was removed from the blood of a non-sensitive (Caucasian) donor, and the red cells were washed once in 0.9 per cent sodium chloride solution. A suspension of approximately 50 per cent was made in a saline-phosphate buffer (tonicity 1.0, pH 7.0).[3] To one portion, 1 part of 0.9 per cent sodium chloride solution was added to 9 parts of suspension; to another portion, 1 part of 5 per cent glucose in 0.9 per cent sodium chloride solution was added to 9 parts of suspension. GSH stability tests were carried out on aliquots of these two suspensions immediately and after incubation for 2 hours and 4 hours at 37° C. After 4 hours, 1 part of 5 per cent glucose in 0.9 per cent sodium chloride solution was added to 9 parts of the suspension which had been incubated without glucose. A GSH stability test was carried out immediately and after further incubation at 37° C for one and three-quarters hours. The results of this experiment are presented in Figure 3. They demonstrate that glucose is required for the protection of GSH by non-sensitive cells, and that even after incubation for 4 hours without glucose, non-sensitive cells quickly regain their capacity to protect GSH when glucose is again made available.

To determine the effect of a number of other substrates on the ability of red cells to protect their GSH, sensitive (by test) and non-sensitive (Caucasian) cells were washed once in 0.9 per cent sodium chloride solution, and the packed cells were resuspended in an approximately equal volume of saline-phosphate buffer. To aliquots of the red cell suspension the following substrates, dissolved in saline-phosphate buffer, were added in the proportion of 1 part of substrate to 4 parts of cell suspension: glucose, 0.100 M; glucose, 0.100 M with methylene blue, 0.00025 M; inosine, 0.100 M; sodium lactate, 0.200 M; sodium pyruvate, 0.200 M; sodium malate, 0.200 M; sodium fumarate, 0.200 M; ribose, 0.100 M; saline-phosphate buffer control (no substrate). GSH stability tests were carried out in the usual manner. The re-

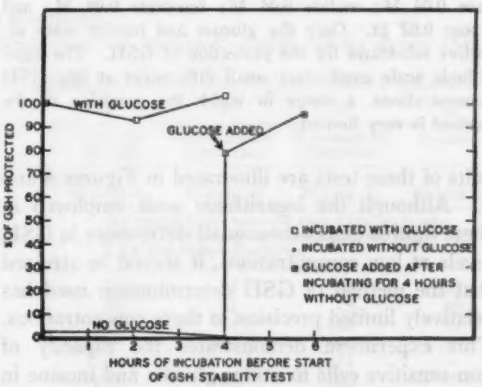

FIG. 3. THE EFFECT OF GLUCOSE ON THE CAPACITY OF NON-SENSITIVE (CAUCASIAN) RED CELLS TO PROTECT THEIR GSH CONTENT

A suspension of washed red blood cells from a non-sensitive (Caucasian) subject was incubated with and without the addition of glucose. At intervals GSH stability tests were carried out on aliquots of the suspensions. The cell suspension without glucose rapidly regained its capacity to protect GSH when glucose was added.

[3] Prepared according to the directions of Ponder (16).

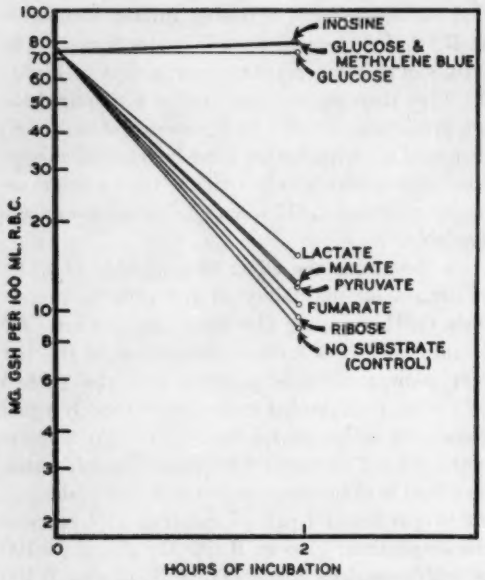

FIG. 4. THE EFFECT OF SUBSTRATE ON THE RESULTS OF THE GSH STABILITY TEST CARRIED OUT ON ERYTHROCYTES FROM A NON-SENSITIVE (CAUCASIAN) SUBJECT

The red cells were suspended in saline-phosphate buffer, pH 7.0. The final concentrations of substrates used were: glucose 0.02 M; glucose 0.02 M with methylene blue 0.00005 M; inosine 0.02 M; lactate 0.04 M; pyruvate 0.04 M; malate 0.04 M; fumarate 0.04 M; and ribose 0.02 M. Only the glucose and inosine were effective substrates for the protection of GSH. The logarithmic scale emphasizes small differences at low GSH concentrations, a range in which the precision of the method is very limited.

sults of these tests are illustrated in Figures 4 and 5. Although the logarithmic scale employed in these figures emphasizes small differences in GSH levels at low concentrations, it should be stressed that the method of GSH determination used has relatively limited precision at these concentrations. This experiment demonstrates the capacity of non-sensitive cells to utilize glucose and inosine in protecting their GSH and their inability to protect significant quantities of GSH in the presence of any of the other substrates tested. Sensitive cells were able to protect their GSH only partially with glucose and inosine substrates.[4] The presence of methylene blue failed to protect significant quantities of GSH.

Because of the impermeability of red cells to phosphorylated sugars (17), and to the enzymes and coenzymes involved in their metabolism, it was necessary to carry out further studies of the enzymatic basis of the protection of red cell GSH in a hemolyzed system.

After establishing optimal conditions, one volume of packed red cells which had been washed once in 0.9 per cent sodium chloride solution was mixed with one volume of a solution containing

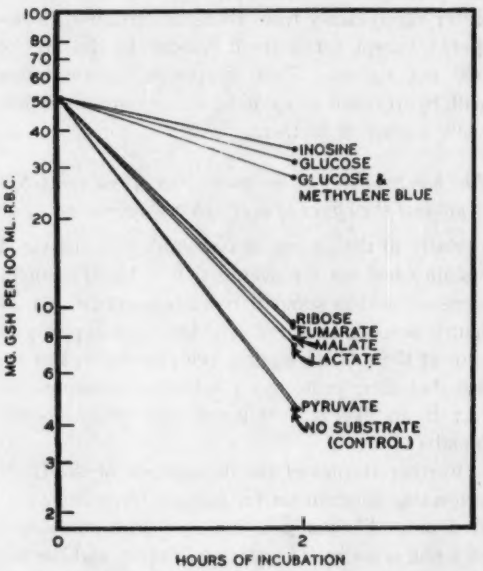

FIG. 5. THE EFFECT OF SUBSTRATE ON THE RESULTS OF THE GSH STABILITY TEST CARRIED OUT ON ERYTHROCYTES FROM A SENSITIVE SUBJECT

The red cells were suspended in saline-phosphate buffer, pH 7.0. The final concentrations of substrates used were: glucose 0.02 M; glucose 0.02 M with methylene blue 0.00005 M; inosine 0.02 M; lactate 0.04 M; pyruvate 0.04 M; malate 0.04 M; fumarate 0.04 M; and ribose 0.04 M. Only the glucose and inosine were effective substrates for the protection of GSH. The greater degree of protection of GSH observed than in the GSH stability test carried out on whole blood is attributed to differences in the properties of the suspended medium. The logarithmic scale emphasizes small differences at low GSH concentrations, a range in which the precision of the method is very limited.

[4] The degree of protection was somewhat better than when the GSH stability test is carried out on whole blood from sensitive individuals (11). This is probably accounted for by the different suspending medium, which, among other things, have a lower pH and higher glucose concentration than blood plasma.

200 mg. per cent GSH and 978 mg. per cent nicotinamide. Nicotinamide inhibits enzymatic hydrolysis of the pyridine nucleotides (18). The mixture was hemolyzed by freezing in a dry ice-alcohol bath and thawing. Aliquots were mixed with aqueous solutions of glucose, glucose-1-phosphate, glucose-6-phosphate, 6-phosphogluconate, fructose-6-phosphate, fructose-1,6-diphosphate, inosine, ribose, ribose-5-phosphate, ATP, TPNH, DPNH, DPN, and distilled water, in the concentrations indicated in Table III. One-ml. aliquots of the resultant mixtures were incubated with 5 mg. of acetylphenylhydrazine at 37° C. These were mixed by shaking at hourly intervals. The GSH concentration was measured in the usual manner at the end of a 4-hour incubation period.

The results of experiments utilizing hemolysates from both non-sensitive (Caucasian) and sensitive donors are presented in Table III. As this table shows, no consistent differences were demonstrated between the GSH stability of hemolysates of sensitive and non-sensitive cells. In the limited observations made, both types of hemolysate protected their glutathione in one or more experiments when glucose-1-phosphate, glucose-6-phosphate, 6-phosphogluconate, fructose-6-phosphate, inosine, fructose diphosphate, ribose-5-phosphate, ATP, DPNH, TPNH, and DPN were used as substrates. Glucose and ribose offered virtually no protection when compared with water. It will be noted that there are certain irregular and sometimes paradoxical results in several of the experiments reported. These involve chiefly results obtained through the use of ribose-5-phosphate and fructose-6-phosphate, both of which were obtained as barium salts. Further investigation demonstrated that excess sulfate ion interferes with GSH protection by hemolysates. Furthermore, the results of phosphate analysis and other studies indicate that our assumption of 100 per cent purity was incorrect. Illustrative of our difficulties in obtaining satisfactory phosphorylated compounds to test in the hemolyzed system was the finding that while the first batch of 6-phosphogluconate used adequately protected GSH, a subsequent lot from the same manufacturer (not used in the experiments reported here) differed in physical appearance from the first lot and not only failed to protect GSH but appeared to destroy it. In view of such difficulties it seems prudent to regard the

TABLE III

GSH stability in hemolysates

Conditions: Each hemolysate preparation contains the following final concentrations: packed cell hemolysate 25 per cent; nicotinamide .02 M; *added* GSH 50 mg. per cent.

Experiment No.		1	2	3	4
Donor		Sensitive (by test)	Non-sensitive (Caucasian)	Non-sensitive (Caucasian)	Sensitive
GSH concentration of hemolysate preparation before incubation		53.4 mg. %	55.8 mg. %	57.4 mg. %	64.0 mg. %
Substrate or coenzymes	Final substrate concentration (100% purity and yield assumed except for TPNH and DPNH)	Per cent of GSH recovered after 4 hrs. incubation of 1-ml. aliquots of hemolysate preparation with 5 mg. acetylphenylhydrazine			
None (control)	0	34	41	24	33
Glucose	.008 M	36	51	26	30
Glucose-1-phosphate	.008 M	—	—	103	90
Glucose-6-phosphate	.008 M	110	105	96	91
Glucose-6-phosphate	.004 M	—	—	71	—
6-phosphogluconate	.008 M	111	106	100	—
Fructose-6-phosphate	.008 M	108	56	57	22
Fructose-1,6-diphosphate	.008 M	63	80	71	63
Inosine	.025 M	96	103	—	—
Inosine	.008 M	—	—	91	85
Ribose-5-phosphate	.008 M	—	104	52	65
Ribose	.008 M	—	—	23	30
DPNH	.0015 M	—	103	—	—
DPNH	.0020 M	—	—	72	83
DPN	.0026 M	—	—	—	50
TPNH	.0007 M	—	48	—	70
TPNH	.0020 M	—	—	68	—
ATP	.009 M	—	—	90	58

results of our study of hemolysates valid only in a qualitative sense.

In addition to our studies carried out at 37° C, attempts were made to demonstrate the difference between hemolysates made from sensitive and non-sensitive cells by incubating them with acetylphenylhydrazine at 25° C. After incubation for 13 hours at this temperature, it was possible to show that the GSH was destroyed in hemolysates to which no glucose-6-phosphate had been added and was protected in the presence of glucose-6-phosphate. However, no difference could be demonstrated between hemolysate made from a non-sensitive (Caucasian) and a sensitive (by test) individual.

DISCUSSION

When drug-sensitive red cells are incubated with acetylphenylhydrazine under standardized conditions, the GSH in these cells is destroyed rapidly. It is apparent from the studies reported here that this destruction of GSH does not depend upon a direct action of acetylphenylhydrazine upon GSH. Oxyhemoglobin, or some other substance in the oxyhemoglobin solution, is required. If oxyhemoglobin itself is the needed substance it is required in concentrations much higher than those which might be expected to suffice if its action were that of a catalyst. It appears most likely that oxyhemoglobin is altered by acetylphenylhydrazine to a form which destroys GSH. The active product has not been identified spectrophotometrically, but it is not methemoglobin or sulfhemoglobin, and, once formed, its activity is not inhibited by cyanide or carbon monoxide.

As the GSH disappears from the red cells, the amount of oxidized glutathione (GSSG) increases. This suggests that the "destruction" of GSH which we observed represents oxidation. After the initial increase in the concentration of red cell GSSG, its concentration also decreased. Although we have not investigated the fate of the GSSG, Flanagan (19) has recently observed that cotton impregnated with silver nitrate turns black when suspended over a blood sample during the glutathione stability test. This finding suggests that the oxidized glutathione may be changed ultimately to hydrogen sulfide.

Since it appears from our findings that the destruction of glutathione observed in red cells actually represents oxidation, it would seem reasonable to examine the enzyme system concerned with glutathione reduction for the abnormality of sensitive red blood cells. Rall and Lehninger (20) have shown that glutathione is reduced in animal tissues through the action of reduced triphosphopyridine nucleotide (TPNH) and "glutathione reductase." Reduced DPN (DPNH) was found to be ineffective. According to the metabolic schemes that have been proposed for the red blood cell, as reviewed by Bartlett and Marlow (21), Granick (22) and Dickens (23) (Figure 6), TPN is reduced at two stages of red cell metabolism. The first of these is the oxidation of glucose-6-phosphate to 6-phosphogluconate. The second is the oxidation of 6-phosphogluconate to 2-keto-6-phosphogluconate. Recently, Carson, Flanagan, Ickes, and Alving (24) have found that dialyzed hemolysates from primaquine-sensitive individuals differ from those of non-sensitive individuals in their lack of capacity to reduce oxidized glutathione when supplied with a glucose-6-phosphate substrate. When a phosphogluconate substrate or TPNH is supplied, however, hemolysates made from drug sensitive cells are as effective as those made from non-sensitive cells in reducing GSSG. This suggests that the glutathione reductase of sensitive cells is intact, but that such cells are deficient in glucose-6-phosphate dehydrogenase activity (Figure 6).

Our observations in intact cells are compatible with the existence of such a metabolic lesion in drug-sensitive cells. They indicate that the striking difference in the GSH levels of sensitive and non-sensitive cells after incubation with acetylphenylhydrazine may well be ascribed to a defect in glucose-6-phosphate oxidation in sensitive cells. It is apparent from the metabolic pathways outlined in Figure 6 that both glucose and inosine may be utilized as substrates for TPN reduction in normal cells. When the GSH of non-sensitive cells is oxidized by acetylphenylhydrazine modified hemoglobin, the GSSG formed could be reduced readily to GSH in the presence of glucose or inosine substrates. If glucose-6-phosphate dehydrogenase activity is reduced in sensitive cells, the pathways of TPN reduction would be interrupted both with glucose and with inosine sub-

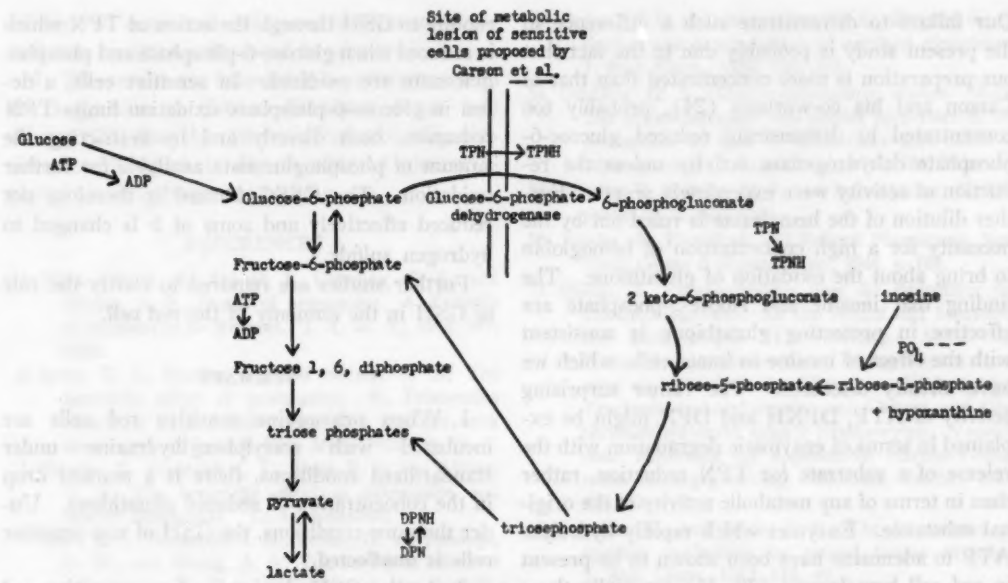

FIG. 6. CARBOHYDRATE METABOLISM IN THE RED CELL: AN ABBREVIATED SCHEME BASED ON SEVERAL SOURCES (21–23, 25)

Most or all of the reactions are reversible under proper conditions.

strates. When the GSH of sensitive cells is oxidized by acetylphenylhydrazine modified hemoglobin, the rate of GSSG reduction would be impaired. Furthermore, if the protection of red cell GSH depended upon TPN reduction, those substrates that are not utilized for TPN reduction should be ineffective in protecting the GSH of either sensitive or non-sensitive cells. Thus lactate, malate, and fumarate, substrates known to be effective in reducing methemoglobin, presumably through a DPN-linked system (26, 27), should be unable to protect GSH either in sensitive or non-sensitive cells. Ribose, which is not metabolized by intact cells (28) should also be inert in the protection of GSH. These predictions were borne out by the experimental data presented in Figures 4 and 5. Glucose and inosine are effective substrates for GSH protection in non-sensitive cells, but in sensitive cells these substrates were less effective. Those substrates, which do not result in TPN reduction, were uniformly ineffective in protecting GSH, both in sensitive and non-sensitive cells.

Although the observations made on suspensions of intact red cells have been helpful in furthering our understanding of the mechanism of drug sensitivity, the scope of the observations has been limited by the impermeability of the red cell membrane to many important metabolic intermediaries. On the other hand, the value of observations we have been able to make on the protection of GSH in hemolysates is impaired by the less physiologic conditions under which they are carried out and by certain technical difficulties that we have described. In the hemolyzed red cells glucose cannot be metabolized (29) presumably due to an absence of ATP and to hydrolysis of the pyridine nucleotides (22). Only by using the phosphoric acid esters of hexoses or ribose as substrates, and by inhibiting coenzyme hydrolysis with nicotinamide, has it been possible to measure the protection of GSH in a hemolyzed system.

Since Carson, Flanagan, Ickes, and Alving (24) have demonstrated a defect in glucose-6-phosphate dehydrogenase activity of sensitive cells, we might have expected hemolysates made from sensitive red cells to fail to protect their GSH when a glucose-6-phosphate substrate was used, while non-sensitive cells might be expected to protect their GSH adequately under the same conditions.

Our failure to demonstrate such a difference in the present study is probably due to the fact that our preparation is more concentrated than that of Carson and his co-workers (24), probably too concentrated to demonstrate reduced glucose-6-phosphate dehydrogenase activity unless the reduction of activity were exceedingly severe. Further dilution of the hemolysate is ruled out by the necessity for a high concentration of hemoglobin to bring about the oxidation of glutathione. The finding that inosine and ribose-5-phosphate are effective in protecting glutathione is consistent with the effect of inosine in intact cells, which we have already discussed. The rather surprising activity of ATP, DPNH and DPN might be explained in terms of enzymatic degradation with the release of a substrate for TPN reduction, rather than in terms of any metabolic activity of the original substance. Enzymes which rapidly hydrolyze ATP to adenosine have been shown to be present in red cell hemolysates (30, 31) especially those in which the stroma was damaged by freezing and thawing (32). Adenosine is metabolized to inosine by red cells (33). DPN has been shown to undergo degradation to adenosine-5-phosphate in the presence of an enzyme, nucleotide pyrophosphatase, which is found in various animal tissues (34). The rapid destruction of DPN which occurs in red cell hemolysates is well documented (35), but we know of no effort to demonstrate the presence of nucleotide pyrophosphatase. If this enzyme were present in red cells, inosine could be formed from DPN as well as from ATP. The utilization of inosine as a substrate for GSH protection has already been discussed above. The effectiveness of fructose-6-phosphate, fructose-diphosphate, and glucose-1-phosphate is probably due to their conversion to glucose-6-phosphate. The effectiveness of 6-phosphogluconate is probably due to its oxidation to 2-ketophosphogluconate with the reduction of TPN.

At the present state of our knowledge it appears to us that the fall in GSH concentration of sensitive cells incubated with acetylphenylhydrazine and the protection of GSH in non-sensitive cells may be explained as follows: upon incubation with acetylphenylhydrazine the oxyhemoglobin of red cells is altered to a product which oxidizes GSH. In normal cells the GSSG formed is reduced rapidly to GSH through the action of TPN which is reduced when glucose-6-phosphate and phosphogluconate are oxidized. In sensitive cells, a defect in glucose-6-phosphate oxidation limits TPN reduction, both directly and by restricting the amount of phosphogluconate available for further oxidation. The GSSG formed is therefore not reduced effectively and some of it is changed to hydrogen sulfide.

Further studies are required to clarify the role of GSH in the economy of the red cell.

SUMMARY

1. When primaquine sensitive red cells are incubated with acetylphenylhydrazine under standardized conditions, there is a marked drop in the concentration of reduced glutathione. Under the same conditions, the GSH of non-sensitive cells is unaffected.

2. As the GSH disappears from sensitive red cells, an increase in oxidized glutathione (GSSG) occurs, suggesting that the disappearance of GSH is due to its oxidation. Subsequently, the amount of GSSG in the red cell also diminishes.

3. When GSH is incubated with acetylphenylhydrazine, no destruction of GSH takes place. The addition of oxyhemoglobin in relatively high concentration is necessary for GSH destruction to be observable. Carboxyhemoglobin, methemoglobin and cyanmethemoglobin are much less effective.

4. Non-sensitive red cells are able to protect their GSH in the presence of glucose or inosine, but not in the presence of lactate, pyruvate, malate, fumarate, ribose, or in the absence of substrate.

5. Sensitive red cells are able to protect only a part of their GSH in the presence of glucose or inosine substrate. The addition of methylene blue does not increase their capacity to protect their GSH.

6. Red cell hemolysates containing nicotinamide and added GSH were able to protect their GSH with glucose-6-phosphate, 6-phosphogluconate, fructose-6-phosphate, fructose-1, 6-phosphate, inosine, ribose-5-phosphate, glucose-1-phosphate, ATP, DPN and TPN substrates. No protection was observed with glucose or ribose substrates or when no substrate was present. In the system

studied, no consistent difference between sensitive and non-sensitive cells could be observed.

7. The results are believed to be consistent with the suggestion of Carson, Flanagan, Ickes, and Alving (24) that the metabolic lesion of drug-sensitive erythrocytes is due to reduced glucose-6-phosphate dehydrogenase activity.

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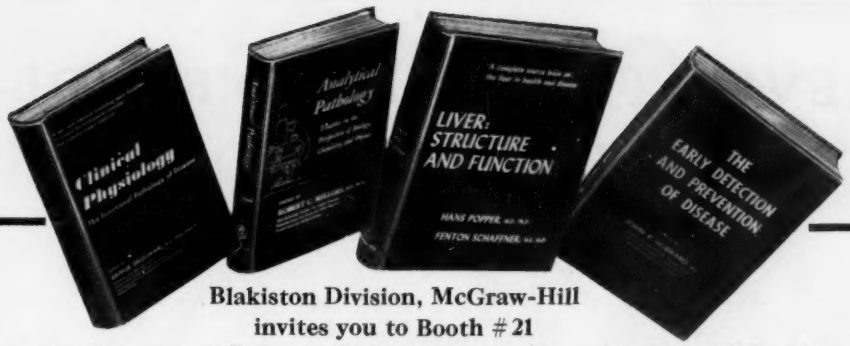

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La protéolyse précoce au cours des anuries hémolytiques du post abortum. G. RICHET et D. ALAGILLE.

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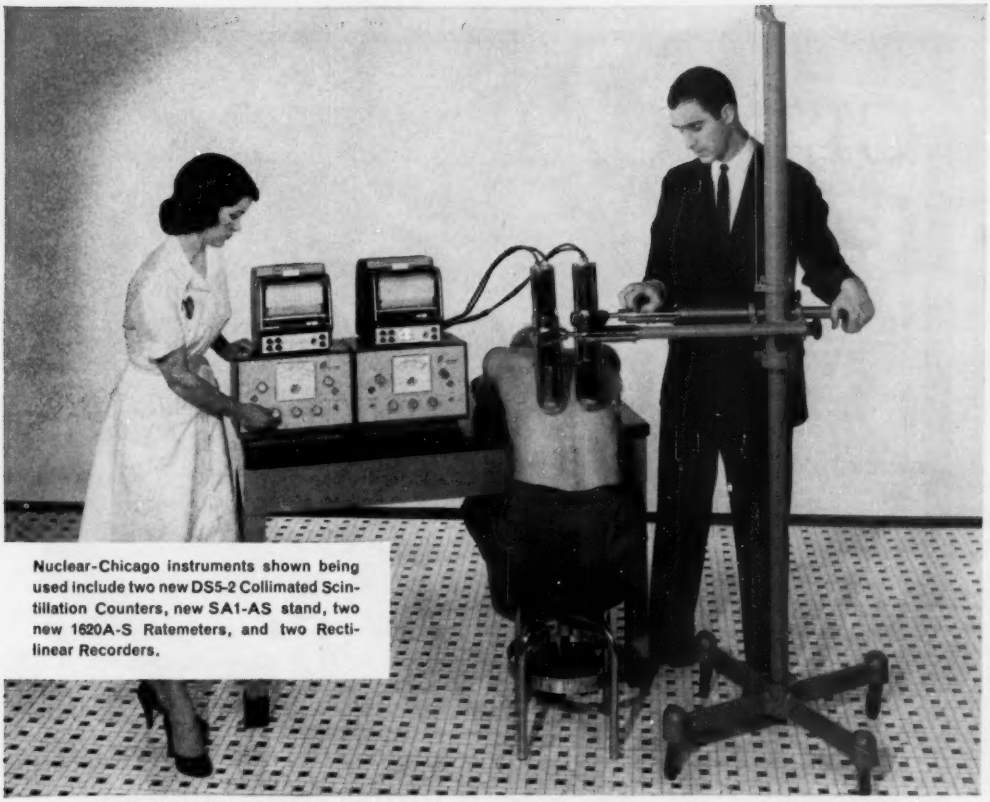

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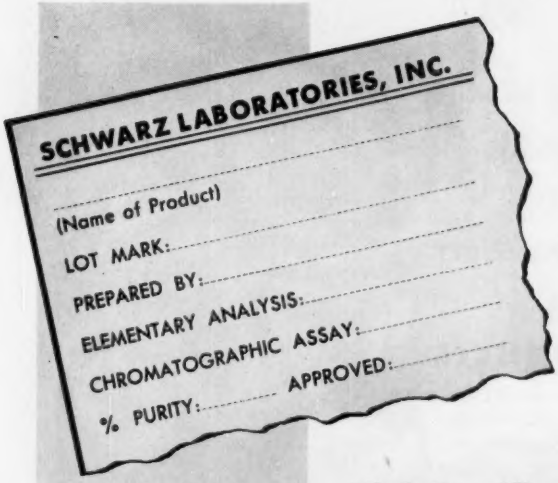

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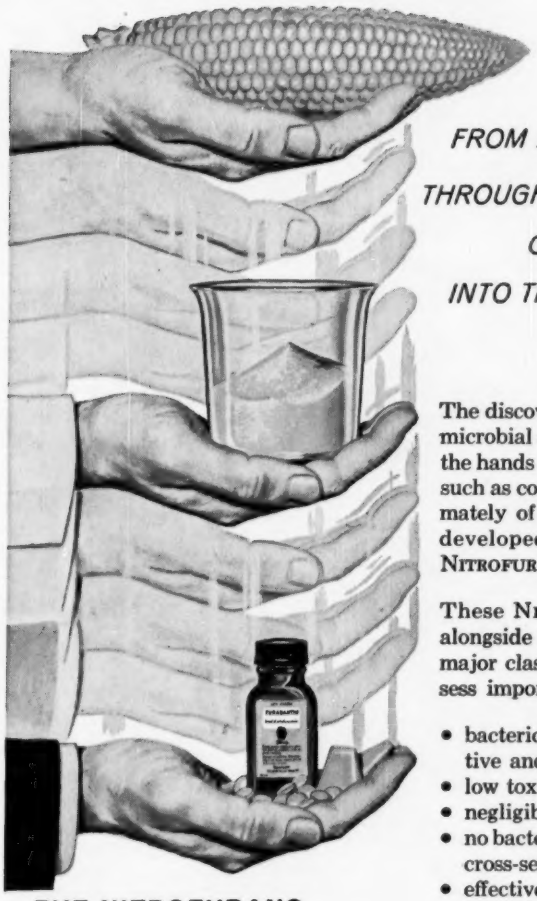

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